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MOLECULAR ASPECT OF SMALL BOWEL ADENOCARCINOMA FOR NEW THERAPEUTIC APPROACHES

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INDICE

ABSTRACT

Small Bowel Adenocarcinoma (SBA) is a rare and aggressive neoplastic disease. Several aspects of SBA carcinogenesis still need to be elucidated, but risk factors and histomorphological similarities seem to indicate that SBA can follow a carcinogenetic development similar to that proposed for colorectal cancer. At molecular level, at odds with adenocarcinoma arising in the large intestine, very few, and fragmented, information is available for SBA. In general, it has been suggested that the two models of colorectal carcinogenesis can be valid also for SBA. For that reason, chemotherapies set up for the cancers of the large intestine have been applied also for SBA. Therefore, since recent studies led to the introduction of EGFR-targeted therapies in colorectal cancer, the treatment with anti-EGFR drugs can be proposed also for SBA patients. In particular, in colorectal cancer patients it has been demonstrated that *KRAS* mutations are correlated with the absence of efficacy of EGFR-targeted therapies, and it has been proposed by few studies to investigate additional markers of the EGFR pathway (*EGFR* gene copy number, *BRAF* and *PIK3CA* mutations, as well as PTEN protein expression), in order to increase the predictive power of the efficacy of anti-EGFR drugs. Information regarding these markers in SBA is quite completely missing.

Primary aim of the present work was the evaluation, in the same cohort, of all the alterations involved in colorectal carcinogenesis, in order to shed light more deeply about the molecular similarity between SBA and colorectal cancer. Second aim of the present work was to investigate in the same cohort of SBA the aforementioned markers involved in the EGFR pathway, in order to verify if the pattern of these alterations could justify the possible introduction of these therapies also in patients affected by SBA.

To do this, for the first aim we investigated β-catenin protein expression by immunohistochemistry (IHC), and *KRAS* and *TP53* mutations by direct sequencing, as well as microsatellite instability (MSI) and allelic imbalance of Chromosome 18q MSI by fragment analysis on genomic DNA extracted from formalin-fixed paraffin-embedded tissue sections. For the second aim we investigated *EGFR* gene status by

Fluorescent in situ hybridization (FISH), *BRAF* and *PIK3CA* mutational status by direct sequencing, as well as PTEN protein expression by IHC, in the same cohort of SBA.

We recruited 40 SBA cases from the Institute of Pathology of Locarno (Canton Tessin, Southern Switzerland) and from three institutions of Northern Italy.

First aim. β-catenin overexpression was observed in 23.6% at nuclear level and in additional 47.3% of cases only at cytoplasmic level, MSI was found in 23.6% of cases, *KRAS* mutations in 43.6% of cases, *TP53* mutations in 29% of cases and allelic imbalance of Chromosome 18q in 75% of cases. All the percentages of alterations and the types of mutations are in line with those identified in the analysis of colorectal cancer patients. Therefore, by the analysis of all these markers in a same cohort, we can confirm that SBA shares the carcinogenetic development with colorectal cancer also at molecular level.

Second aim. We identified a copy number gain of *EGFR* gene in 57.5% of cases, *BRAF* and *PIK3CA* mutations in 2.5% and 10.5% of cases respectively, and PTEN loss of expression in 25.6% of cases. Also for the EGFR pathway analysis, percentages of alterations and types of mutations found in SBA are in line with colorectal cancer, even if we did not detect the classical V600E change in the *BRAF* gene (where, on the contrary, we found a rare mutation, the G596R change). Taking into account the molecular algorithm proposed for the administration of EGFR-targeted therapies in colorectal cancer patients, if we look only at *KRAS* mutations, we can propose the administration of EGFR-targeted therapies to about 60% of patients (i.e.: *KRAS* wild-type cases), and to 23% of cases if we base our evaluation on the whole EGFR pathway (i.e.: cases showing, at the same time, *EGFR* copy number gain, *KRAS*, *BRAF* and *PIK3CA* wild-type sequences, and PTEN normal expression). The treatment with anti-EGFR therapies of a SBA patient of our cohort who developed a metastatic lesion confirmed the relevance of the molecular characterization of the tumor to predict the response to these therapies.

In conclusion, our analyses of SBA confirm the feeling that the mechanisms of carcinogenesis of such disease are superimposable with those proposed for colorectal cancer. Therefore, the hypothesis that therapeutic protocols valid for the large intestine can be applied also to SBA patients is supported. As a consequence, the targeted therapies recently introduced in colorectal cancer can be proposed for SBA patients, pending tumor molecular characterization as demonstrated by our case report.

INTRODUCTION

1.1 Cancers of the small intestine

The small intestine represents the longest part of the digestive tract, making up 75% of the length (about 6 m long and 4 times as long as the large intestine) and 90% of the absorptive surface area of the gastrointestinal tract (Figure 1.1). It has three sections: duodenum, jejunum and ileum (Figure 1.2). Malignant tumors of the small intestine are rare all over the world (Hamilton & Aaltonen, 2000), especially with respect to tumors arising in the other portions of the gastrointestinal tract, with a global incidence of less then 1.0 per 100,000 population (Curado et al, 2009). Cancers of the small intestine, including adenocarcinoma, carcinoid, lymphoma and sarcoma (the two latter are rarer than adenocarcinoma and carcinoid) account for only 0.42% of total cancer cases and 2.3% of cancers of digestive system in the United States (Jemal et al, 2009). Mortality of the cancer is even lower, accounting for only 0.2% of the total cancer deaths in the United States (Jemal et al, 2009).

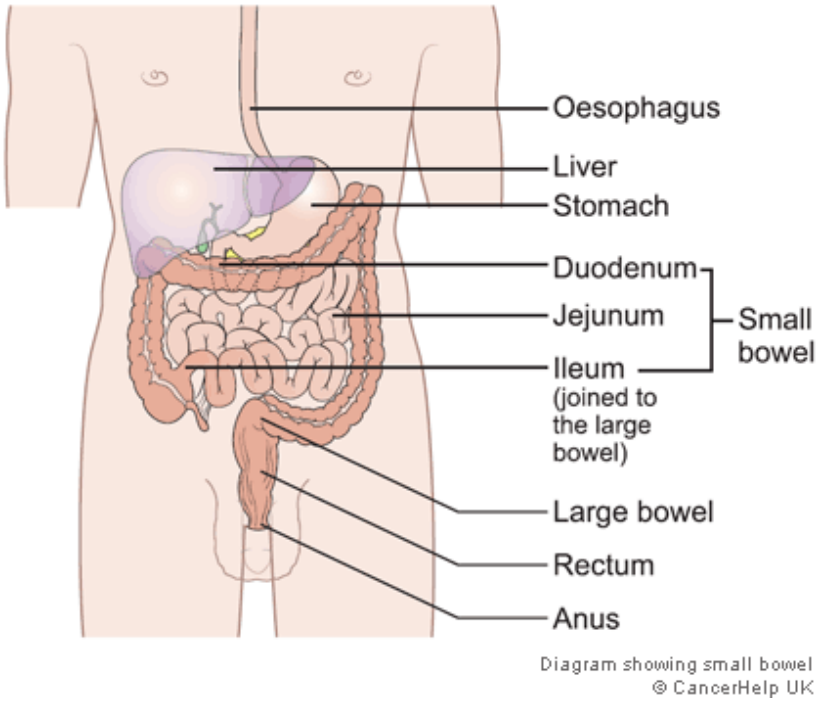

Figure 1.1: Gastrointestinal tract.

Approximately 30%-40% of the cancers observed in the small bowel are adenocarcinomas (SBA), a percentage much lower than the proportion in the colon where the overwhelming majority is adenocarcinomas (Haselkorn et al, 2005; Bilimoria et al, 2009; Schottenfeld et al, 2009). The incidence rates for SBA are 0.5-1.5 per 100,000 in men and 0.2-1.0 per 100,000 in women. High incidence rates are observed in black people in several regions of the United States: more than 2 per 100,000 men and about 1.25 per 100,000 women in the regions included in the Surveillance, Epidemiology and End Results (SEER) program. High rates are also observed in Hawaii and in New Zealand. On the contrary, the lower incidence rates can be found in India, Romania and in other countries of Eastern Europe (Neugut et al, 1998; Negri et al, 1999). From 1975 to 2000, it seems that the incidence rates are increased of 50% in several countries, but not in the United States (De Launoit al, 2005). More than 50% of SBA arise in the duodenum, while ileum represents the rarest affected region, with the exception of patients affected by Crohn's disease.

Mean age of SBA occurrence is between 55 and 65 years, even if a few cases have been described in younger patients, especially in cases belonging to families with a inherited history of colorectal cancer, or with Cronh's disease (Lashner et al, 1992).

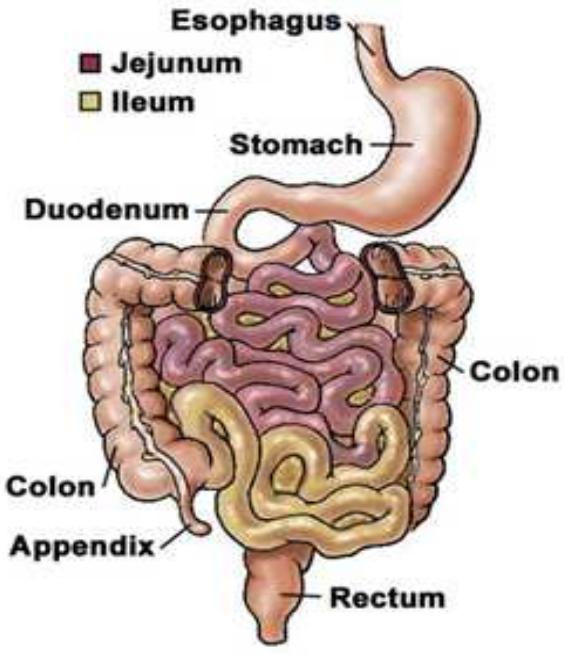

Figure 1.2: Small intestine.

1.2 Risk factors

The reason for the much lower incidence of small intestinal cancer than of colorectal cancer is largely unknown but has been hypothesized to be related to several mechanisms. The much quicker transit time of food in the small intestine than in the large intestine (because peristaltic ring contractions in the small intestine occur with greater frequency than in the colon) may result in shorter time of exposure of its mucosa to carcinogens. The small intestine has much lower bacterial load, thus has decreased concentration of potential carcinogens from bile acid breakdown (Arber et al, 1997). Studies also demonstrate that the small intestine generates less endogenous reactive oxidative species than the colon does, which may lead it to handle oxidative stress more effectively than the colon thus resulting in less oxidative damage during times of exposure to oxidant stress (Sanders et al, 2004).

Inflammatory bowel disease includes Crohn's disease and ulcerative colitis, two clinically related but histologically distinct diseases. Crohn's disease is a recognized risk factor for SBA, with relative risks reported as high as 60 (Neugut et al, 1998; Pan et al, 2011). A meta-analysis showed a relative risk of 33.2 (95% CI: 15.9-60.9) for SBA in patients with Crohn's disease (Canavan et al, 2006). Extended duration of the disease, distal jejunal and ileal location, male sex, small bowel bypass loops, chronic fistulous disease, young age of diagnosis and occupational hazards or exposure to halogenated aromatic compounds with aliphatic amines, asbestos and solvents are suggested to be associated with an increased risk of SBA in patients with Crohn's disease (De Launoit al, 2005; Feldstein et al, 2008). Ulcerative colitis has been shown to be associated with an increased risk of colorectal cancer, hepatobiliary cancer, nonmelanoma skin cancer and leukemia (Winther et al, 2004; Hemminki et al, 2008). However, it is unclear whether patients with ulcerative colitis have an increased risk also for SBA (Bernstein et al, 2001; Hemminki et al, 2008).

Celiac disease is an inflammatory small intestinal disorder characterized by the inability of the small intestine to deal with the gluten fractions of cereals such as wheat, barley and rye; its prevalence is nearly 1% of general population (Pan et al,

2011). The risk of SBA in patients with celiac disease is increased many-fold as compared with the risk in the general population (Green and Cellier, 2007) with reported relative risks between 60 and 80 (Green et al, 2003). SBA is most often located in the jejunum and is more likely to develop as an adenoma-carcinoma sequence than as dysplasia in flat mucosa (Green and Rampertab, 2004).

Although the prevalence of adenomas in the small intestine is much lower than their prevalence in the colon, it is suggested that the adenoma-carcinoma sequence is as significant in the small intestine as in the large intestine (Sellner, 1990). As in the colon, adenoma in the small intestine appears to be a precursor of adenocarcinoma (Gill et al, 2001). A large fraction of villous adenomas of the small intestine has been shown to progress to malignancy (Bjork et al, 1990).

Familial adenomatous polyposis (FAP) is an autosomal dominant genetic disorder caused by mutations of the APC gene on the long arm of chromosome 5 (Groden et al, 1991). Most patients diagnosed with FAP have multiple adenomas in the small bowel, usually in the duodenum (Bertoni et al, 1996) and these patients are at increased risk of SBA, especially duodenal cancer (Lepisto et al, 2009). The prevalence of duodenal adenomatosis in FAP patients are 50%-90% and 3%-5% of these patients develop duodenal cancer (Kadmon et al, 2001).

The demonstration of a geographical correlation between rates of SBA and colorectal cancer (Haselkorn et al, 2005) suggests a common aetiology. Various studies have shown that the risk of SBA following primary colorectal cancer were elevated; in addition, in those diagnosed with primary SBA, there was a 4 to 5-fold risk of developing colorectal cancer (Murray et al, 2004; Scelo et al, 2006; Lagarde et al, 2009). These studies suggest etiological similarities between adenocarcinomas of the small intestine and of the colon-rectum but, to date, potential common carcinogenic agents have not been elucidated in analytic epidemiological studies. Dietary factors have been suggested to be related to the risk of SBA. A study of SBA mortality and food data by WHO showed correlations with daily consumption of animal fat and animal protein (Lowenfels and Sonni, 1977). A case-control study of 430 SBA cases

and 921 controls observed two-to three-fold increases in SBA risk with frequent intake of red meat and salt-cured/smoked foods but no association with alcohol consumption (Chow et la, 1993). Another case-control study of 36 cases with SBA and 998 population controls also reported a significant increase in risk associated with frequent intake of foods rich in heterocyclic aromatic amines (based on the combined intake of fried bacon and ham, barbecued and/or smoked meat and smoked fish) in males only and with total sugar intake (Wu et al, 1997). A hospital-based case-control study (Negri et al, 1999) found an increased risk of SBA among the highest consumers of red meat and of refined carbohydrates, while a decreased risk was associated with consumption of fish and vegetables.

1.3 Clinico-pathological data

Symptoms predicting the insurgence of SBA depend on the dimension and on the site of the lesion. The majority of these tumors are characterized by aspecific symptoms, able to be perfectly understood only when the disease is at advanced stage. This unlike factor leads to a late diagnosis, and as a consequence the prognosis is often severe. A late diagnosis of 6-8 moths is a common event for two additional reasons:

- SBA can be hardly detected by the endoscope, especially when it occurs in the duodenum;
- X-ray exam is not the preferred methods to identify these lesions (Dabaja et al, 2004).

SBA in the ileum and jejunum is characterized by abdominal pain, nausea, vomiting, weight loss (Schottenfeld et al, 2009). Duodenal SBA has different clinical features, especially due to the fact that lumen is larger than in the ileum or jejunum (Longacre et al, 1990).

1.4 Pathological features

Duodenal SBA is typically polypoid with a necrotic central area (Figure 1.3). Very often an adenomatous component is still present. SBA arising near Papilla of Vater is usually smaller than that arising in the other regions of the small bowel, and appears to be a sort of nodule in the duodenum wall (Longacre et al, 1990).

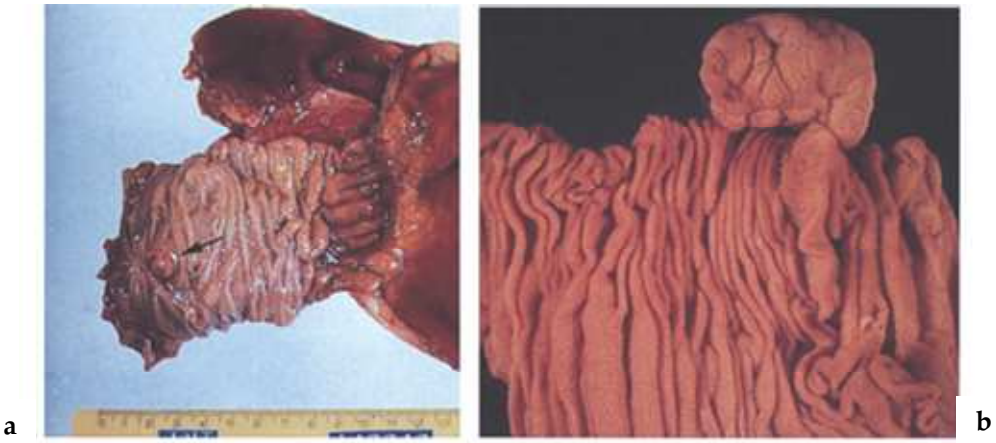

Figure 1.3: Duodenal SBA with the typical polypoid aspect.

SBA of the ileum or jejunum is usually identified at higher stage with respect to duodenal SBA, and therefore the typical appearance is represented by an infiltrative and ulcerated mass protruding in intestinal lumen. In the majority of cases sierosa is infiltrated (Bridge et al, 1975) (Figure 1.4).

SBA occurring in patients affect by Crohn's disease is difficult to be detected because of the presence of deep ulcerative lesions, generally undistinguishable from an inflammatory disease (Horton et al, 1994).

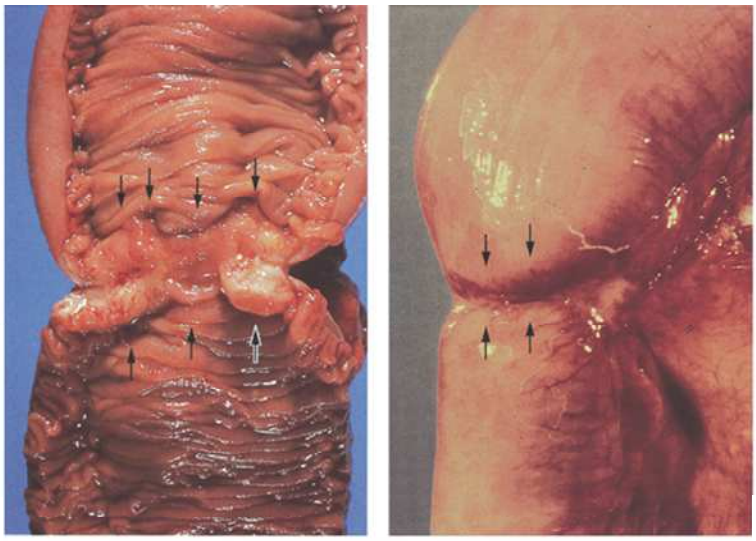

Figure 1.4: SBA with intestinal wall infiltration. **a:** lumen; **b:** sierosa. Arrows indicate intestinal wall infiltration

At histological level, SBA (Figure 1.5) is similar to adenocarcinoma of the colon-rectum.

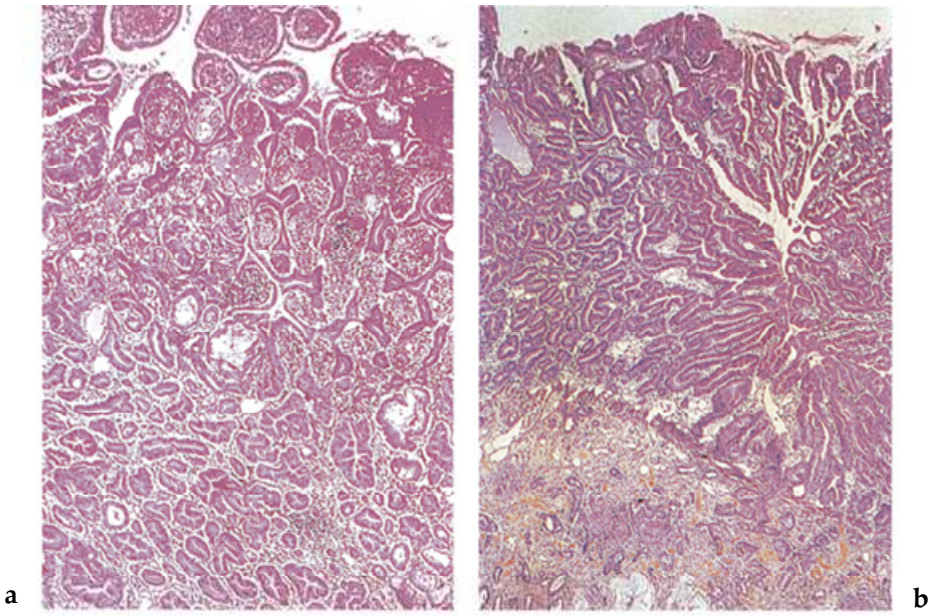

Figure 1.5: Two examples of microscopical appearance of SBA.

1.5 Stage and grade

Classification criteria subdivide SBA in well, moderately and poorly differentiated, thus identifying tumor grade. Well differentiated tumors (G1) (Figure 1.7) are characterized by glandular structures in more than 95% of tumor mass, moderately differentiated cases (G2) between 50 and 95% (Figure 1.8), poorly differentiated tumors (G3) by less than 50% (Figure 1.9). Undifferentiated tumors have only less than 5% of glandular structures (Schlemper et al, 2000). Mucinous adenocarcinoma are considered G3 cases.

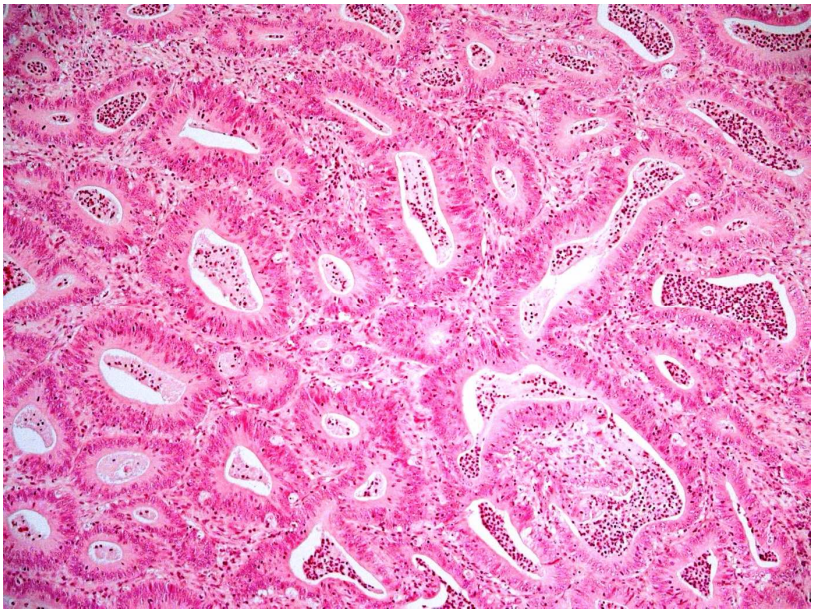

Figure 1.7: Well differentiated SBA (G1).

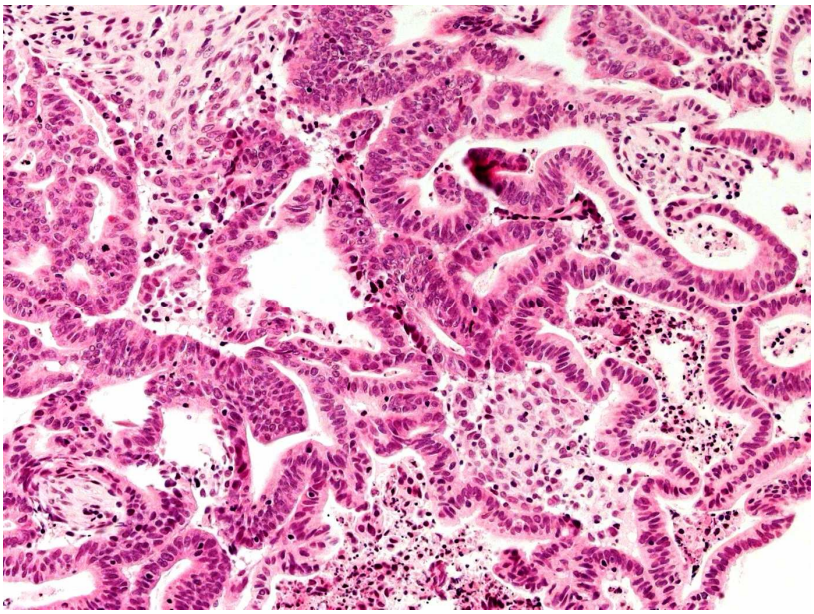

Figure 1.8: Moderately differentiated SBA (G2).

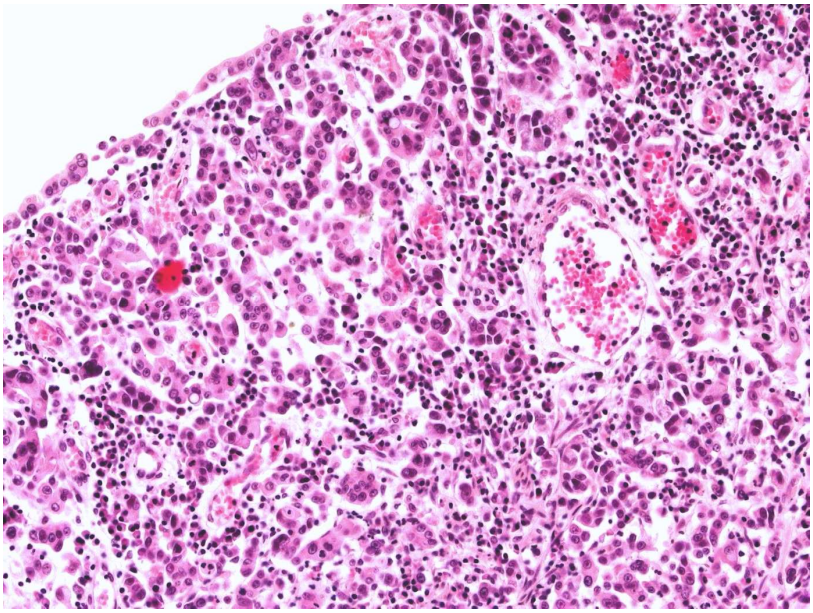

Figure 1.9: Poorly differentiated SBA (G3).

In the presence of tumor heterogeneity, that means that different components coexist in the same tumor, the case is classified on the basis of the higher tumor grade component. However, if poorly differentiated cells are present only at the invasive margin, this feature is not sufficient to classify the tumor as G3.

Tumor staging is similar to that applied for colorectal cancers. UICC classification is based on tumor size and infiltration (T), regional lymph nodal status (N) and presence of distant metastasis (M). This system, also named as TNM, was lastly modified in 2010 and is generally accepted.

The tumoral lesion is considered as adenocarcinoma also in absence of muscolaris mucosa invasion, at odds with criteria valid for colorectal cancer, because in the small bowel lymphatic vessels are present very close to the epithelium, while in the large intestine they are localized in the muscolaris mucosa. Therefore, the possibility of tumoral cells to invade regional lymph nodes is higher in small bowel than in colon-rectum.

1.6 Prognosis

According to the data from US SEER for the period 1992 to 2005, the median 5-years relative survival was 28.0% for SBA in general and 32.5% for patients who underwent resection. This difference emphasizes the benefit of tumor resection on patients' follow-up. Overall, only a minor fraction of patients can survive to SBA, and the cause of the severity of such a neoplastic disease can be principally ascribed to the delay of disease discovery (Dabaja et al, 2004). Although other cancer sites have demonstrated higher long-term survival rates due to novel adjuvant therapies over the last two decades, the US data from 1985 to 2000 showed no significant change in long-term survival rates for SBA (Bilimoria et al, 2009). Another study observed an improvement in survival rates in England, Wales and Scotland over the time period of 1975 to 2002 but the changes were not statistically significant because of the small number of patients (Shack et al, 2006). A Swedish study found 5-years survival rates of 39% for duodenal adenocarcinoma and 46% for jejuno-ileal adenocarcinoma (Zare et al, 1996). Earlier tumor stages at diagnosis (stage I and II), small tumor size and curative resection have been identified as factors for favorable overall survival, whereas poorly differentiated tumors, lymph node involvement or metastasis and distant metastases as factors predicting poor prognosis (Wu et al,

2006; Hamilton et al, 2009). However, the involvement of regional lymph nodes as prognostic factor is still debated, as other studies reported absence of worse survival for advanced cases with respect to patients with a localized disease (Willet at al, 1993; Agrawal et al, 2007).

Primary relapse events in SBA patients are the loco-regional recurrence or liver metastasis. More rarely, peritoneal carcinomatosis can occur. Usually, relapse develops in the first 2 years after tumor resection (Pilati et al, 2001; Bilimoria et al, 2009).

1.7 Clinical treatment

Primary treatment is surgery, adopted whenever possible. Duodenal lesions are resected on the basis of Whipple's indications. However, due to the late diagnosis, very often tumors cannot be resected. A good estimation is that only 50-60% of patients with SBA can be radically resected (Bilimoria et al, 2009).

Tumor relapse, due either to local recurrence or to the presence of distant metastatic lesions, therefore represents the main cause of death for SBA patients. As a consequence, major efforts have been done for the identification of the best chemotherapy. At the moment, however, a specific and standard protocol for the treatment of SBA has not been established yet. In general, oncologists prefer to apply the protocols set-up for cancers arising in other district of the gastrointestinal tract, such as for gastric and, especially, colorectal cancer.

The use of cisplatin turned out to be quite inefficacious (Ono et al, 2008; Suenaga et al, 2009). On the contrary, treatments with good results in SBA patients include oxaliplatin or irinotecan in conjunction with 5-fluorouracil (Overman et al, 2008). In addition, it has been demonstrated that the combination of 5-fluoruracil, doxorubicin and mytomicin C is active and well tolerated in advanced cases (Gibson et al, 2005).

However, all these combinations did not lead to a substantial benefit for SBA patients. Therefore, it is of particular interest the introduction of new therapeutic approaches for such a disease. In this category, we can easily include targeted

therapies, which turned out to be effective in colorectal and gastric cancer. But the possibility to introduce these therapies requires an extensive knowledge of the disease at molecular level, at least for the targets of these new compounds.

1.8 Molecular data

The data concerning the molecular characterization of SBA are few and fragmented, especially due to the low frequency rate of such a disease. In the majority of cases, only few patients are included in the analyses, and usually only few markers are investigated in the same cohort of cases. The most studied markers are those involved in colorectal carcinogenesis, i.e.: microsatellite instability, APC, KRAS, allelic imbalance of Chromosome 18q, and TP53.

1.8.1 Microsatellite instability

Since SBA can develop in Hereditary Non-Polyposis Colorectal Cancer (HNPCC) families, a syndrome characterized by a not functioning DNA mismatch repair system which leads to the accumulation of errors in all over the genotype and especially in small regions named microsatellites, the status of microsatellite instability (MSI) has been investigated. The majority of studies agrees in considering around 10% the fraction of SBA with MSI, a percentage in keeping with what observed in colorectal carcinogenesis (Keller et al, 1995; Svrcek et al, 2003). Another study found a higher percentage of MSI (35%) (Overman et al, 2010).

1.8.2 APC-β-catenin pathway

Since SBA can occur also in FAP patients, the occurrence of *APC* mutations has been studied. The *APC* gene is a tumor suppressor gene encoding for a large multidomain protein that plays a relevant role in the wnt-signalling pathway and in intercellular adhesion. In the normal cells, APC is able to form a multiprotein complex with GSK-3β and axin. This complex binds to β-catenin, which in turn is phosphorylated by GSK-3β and subsequently degraded by the proteasome pathway (see Figure 1.10). In

tumoral cells, when *APC* (as well as β-catenin or axin) is mutated, the multiprotein complex cannot be formed and, therefore, β-catenin accumulates into the cytoplasm and translocates into the nucleus, where activates Tcf factor, causing transcription of target genes (involved in different cellular processes), such as *c-myc*. {4991}. In the literature it seems that *APC* mutations are a rare event in SBA carcinogenesis, at odds with colorectal cancer, where *APC* is mutated in about 80% of sporadic cases (Abrahams et al, 2002; Svrcek et al, 2003).

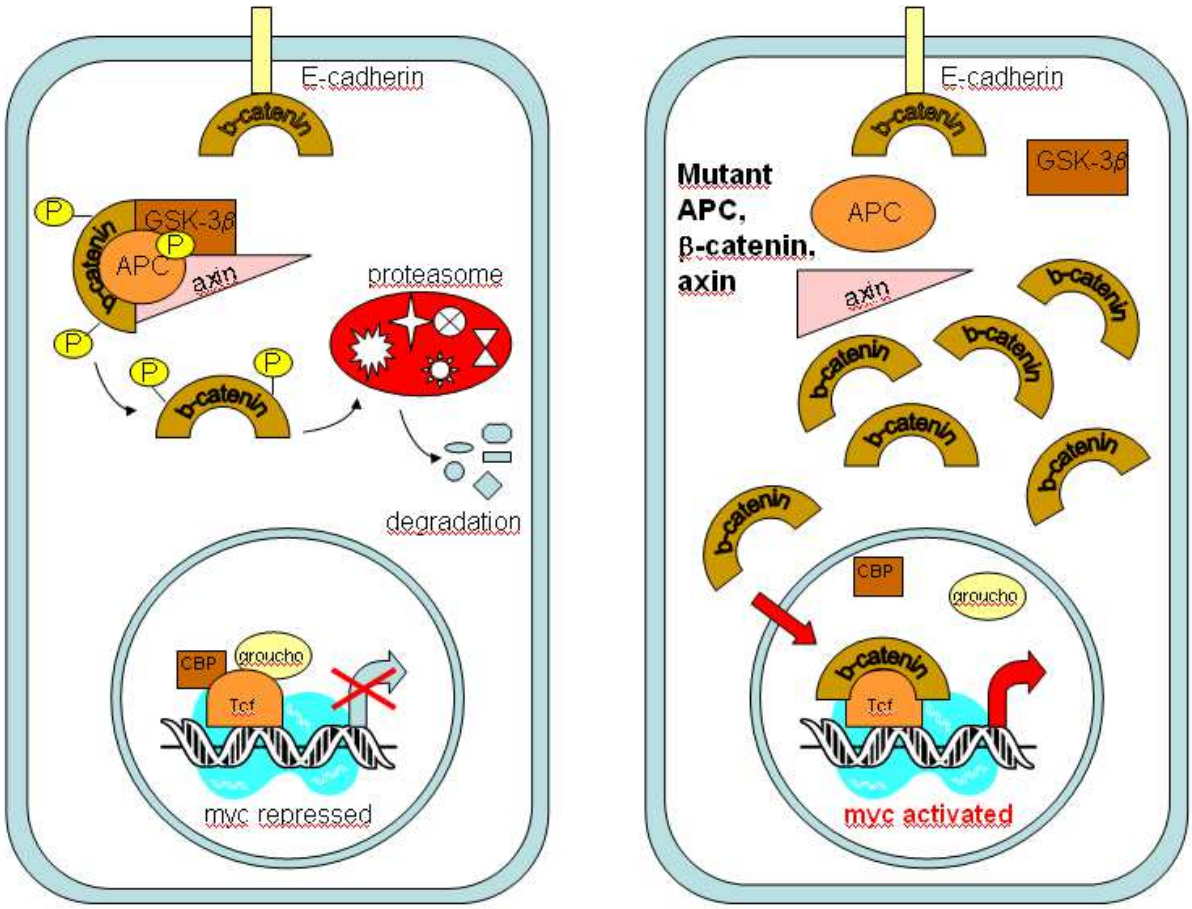

Figure 1.10: APC/β-catenin pathway and myc overexpression in normal (left) and tumoral (right) cells.

1.8.3 KRAS

KRAS gene encodes for a membrane-bound 21 kd protein involved in G protein-mediated signal transduction. KRAS protein can acquire transforming potential secondary to a point mutation in hot spot codons, primarily codons 12 and 13, which prevent the inactivation of GTP and result in continuous KRAS protein activation. In

SBA the percentage of *KRAS* mutation occurrence is superimposable with that found in colorectal cancer (30-40%) (Sutter et al, 1996; Younes et al, 1997). In particular, it has been proposed that *KRAS* mutations are limited to the tumors arising in the duodenum, since have not been observed in tumors of ileum or jejunum (Younes et al, 1997).

1.8.4 Allelic imbalance of Chromosome 18q

An old study reported the loss of the long arm of Chromosome 18q in SBA (Hahn et al, 1996). Mapping to this chromosomal bands are three tumor suppressor genes mainly involved also in colorectal cancer: *Deleted in Colon Cancer (DCC)*, *SMAD4* (previously named *DPC4*, as *Deleted in Pancreatic Cancer locus 4*) and *SMAD2*. The DCC protein plays a relevant role in the regulation of cell-cell and cell-matrix adhesion, while SMAD proteins are involved in cell proliferation and apoptosis. In SBA, the loss of this region can be observed in a wide range of cases, from 15 to 80% of analyzed patients (Bläker et al, 2002; Bläker et al, 2004), similarly to what observed in colorectal cancer.

1.8.5 TP53

The *TP53* gene encodes a 393-aminoacid nuclear phosphoprotein, which negatively controls the cell cycle through transcriptional activation of WAF1/CIP1 gene and through bcl-2 and Bax binding in response to a variety of stress signals including DNA damage as well as hypoxia, radiation exposure, drug exposure. *TP53* mutations, especially occurring in the DNA binding domain (encoded by exons 5-8), represent the main mechanism of TP53 inactivation in cancer. Studies investigating *TP53* mutations in SBA reported their detection in 20-53% of cases, a percentage in keeping with colorectal carcinogenesis (Lane, 1994; Abrahams et al, 2002; De Launoit et al, 2004).

1.8.6 Molecular models of colorectal carcinogenesis

Intensive screening for genetic alterations led to the identification of two major types of colorectal cancer, that are distinct by their carcinogenic process (Figure 1.11). One is characterized by normal caryotype, normal DNA index (Houlston et al, 2001) and genetic instability at microsatellite loci and is called MSI-positive cancer (Ilyas et al, 1999). The second one, valid for more than 90% of sporadic CRC and firstly proposed by Vogelstein's group (Fearon and Vogelstein, 1990), suggests that APC (or, better, the APC-β-catenin pathway) represents the initial mutational event that determines hyperplastic proliferation and then early adenoma formation. The stage of late adenoma is achieved with *KRAS* mutation. Loss of tumor suppressor genes at chromosome 18q and mutations in *TP53* gene lead to carcinoma in situ (Laurent-Puig et al, 1999) and then to metastasis.

Since in SBA the percentages of alterations of these markers are similar to those observed in colorectal cancer, it is generally accepted that the two models proposed for colorectal cancers are valid also for SBA and, therefore, that SBA carcinogenesis mirrors that of the colon-rectum.

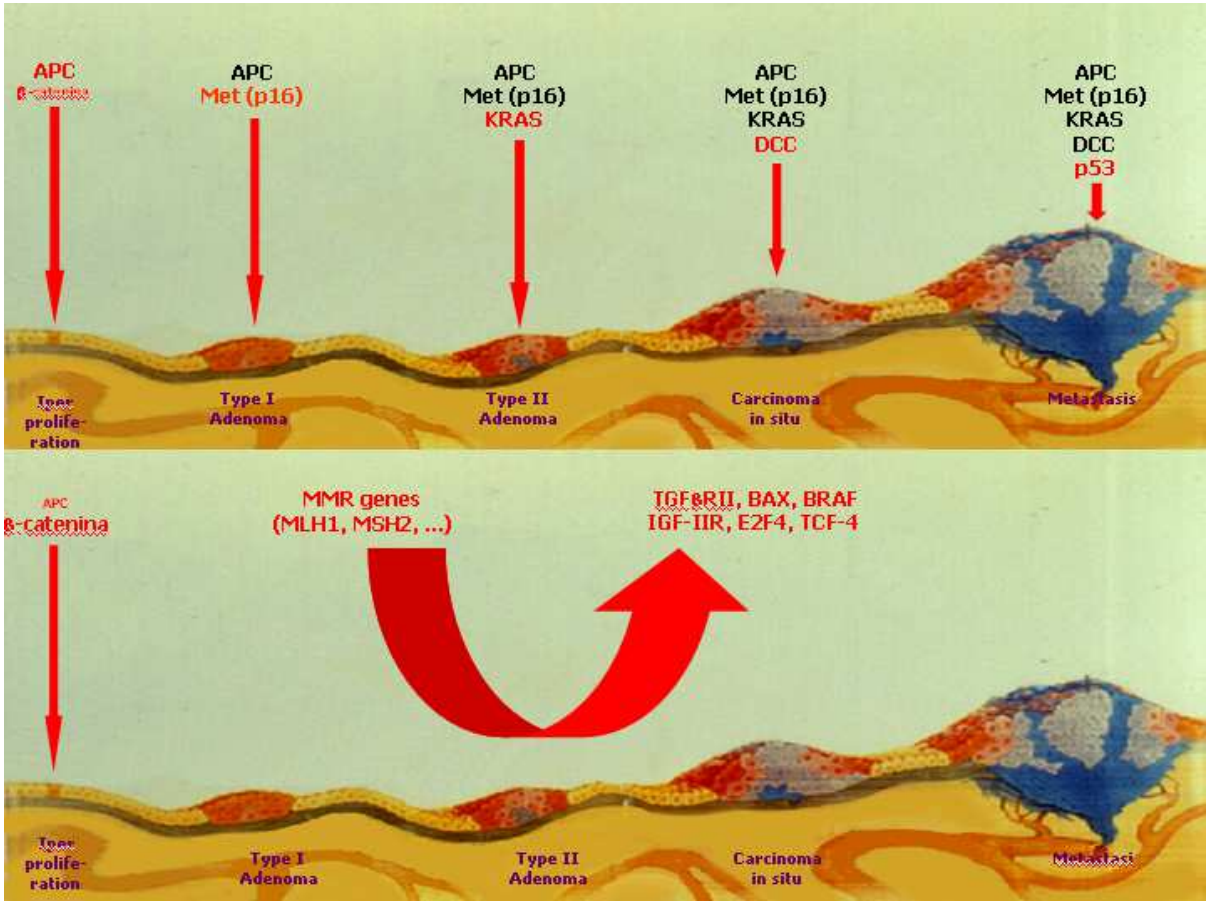

Figure 1.11: Schematic models of colorectal carcinogenesis as proposed by Vogelstein and colleagues

However, these markers have not been introduced in clinical practice in the management of colorectal cancer patients, where, on the contrary, recent evidence pointed out to the relevance of the EGFR pathway.

1.8.7 **EGFR in colorectal cancer**

The EGFR signaling pathway is thought to play a pivotal role in tumor growth and progression of various cancers, including CRC. The *EGFR* gene encodes for a 170 kDa transmembrane receptor with intrinsic tyrosine kinase activity belonging to the ErbB family of receptor TKs [that includes ErbB1 (EGFR or HER1), ErbB2 (HER2), ErbB3, and ErbB4] (Figure 1.12).

EGFR binds to, and then is activated, by several ligands, leading to receptor dimerization, which in turn is able to transmitting the mitogenic signaling through

several pathways into the nucleus by regulating several transcription factors. EGFR is involved in the control of the expression of genes relevant for inhibition of apoptosis and for tumor cell proliferation and survival, migration, adhesion and angiogenesis (Woodburn 1999; Arteaga, 2001; Talapatra et al, 2001; Venook, 2005).

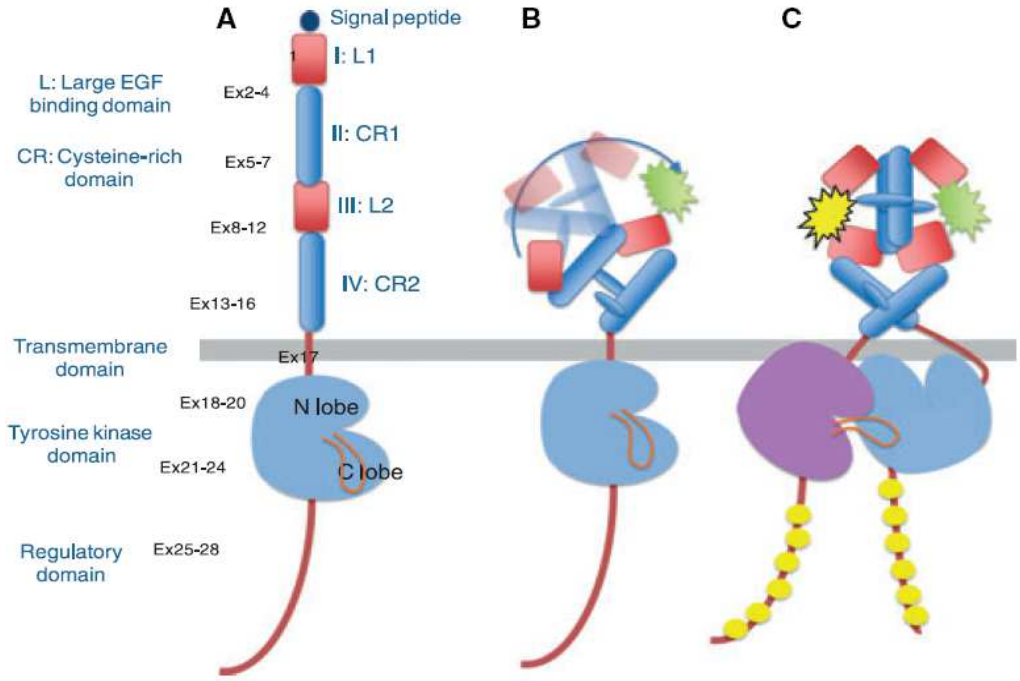

Figure 1.12. Structure of the EGFR protein (A), activation (B) and dimerization by ligand binding (C) (Mitsudomi and Yatabe, 2009).

In colorectal cancer, the main mechanism of EGFR deregulation is represented by protein overexpression following gene amplification or at least an increase of the gene copy number.

1.8.8 EGFR downstream cascade

The two main pathways activated by EGFR are the RAS-RAF-MAP kinase pathway, mainly involved in cell proliferation, and the PI3K-PTEN-Akt pathway, mainly involved in cell survival and escaping from apoptosis. In these two pathways, some members are more deeply involved in colorectal cancer development: KRAS, BRAF, PI3K and PTEN (Figure 1.13).

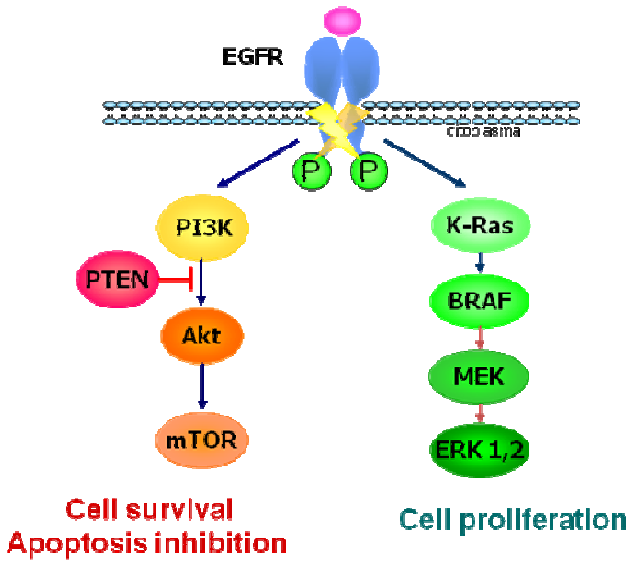

Figure 1.13. Schematic representation of the two main pathways of EGFR downstream cascade.

KRAS. See paragraph 1.8.3.

BRAF. *BRAF* gene encodes for a RAS effector belonging to the RAF family of Ser-Thr kinase proteins. *BRAF* gene product is recruited to the plasma membrane upon binding to RAS-GTP, and represents a key point in the signal transduction through the MAP kinase pathway. The typical *BRAF* alteration, leading in turn in the constitutive activation of BRAF itself and of the MAP kinase pathway, is represented by point mutations, occurring in the vast majority of cases in colorectal cancer (>90%) at codon 600 (with the typical V600E change) (Davies et al, 2002). In colorectal cancer, *BRAF* mutations are frequently found in sporadic cases characterized by MSI, and are mutually exclusive with *KRAS* mutations (Rajagopalan et al, 2002). At the moment, no data concerning the involvement of BRAF in SBA cancerogenesis have been published.

PI3K. Phosphatidylinositol 3-kinases (PI3Ks) belong to the lipid kinases family that regulates the signal transduction (Vivanco et al, 2002). Activation of PI3Ks results in the production of the second messenger phosphatidylinositol (PI) 3,4,5 trisphosphate (PIP_3) from PI 4,5 bisphosphate (PIP_2). PIP_3, through AKT activation, drives various

downstream pathways involved in the regulation of several cellular functions including cellular growth, transformation, adhesion, apoptosis, survival and motility (Yuan et al, 2008).

Only PI3K proteins that contain the catalytic subunit p110α and its associated regulatory subunit p85 (that belongs to the class IA protein) are involved in tumorigenesis (Samuels et al, 2004). The p110α subunit is encoded by *PIK3CA*, which in tumors is frequently iperactivated following point mutations in hot spot codons located in exons 9 and 20.

In colorectal cancer, *PIK3CA* mutations occur in about 10-30% of cases (Samuels et al, 2004), while their occurrence in SBA has never been investigated.

PTEN. PTEN is a tumor suppressor gene that encodes for a 403-amino acid protein that possesses both lipid and protein phosphatase activities. Its typical function consists of dephosphorylation of PIP_3 and PIP_2, thus preventing AKT phosphorylation, and maintaining it in its inactive form, thus counteracting the role of PI3K proteins. PTEN is therefore involved in inhibition of cell cycle progression, induction of cell death, modulation of arrest signal and stimulation of angiogenesis by influencing vascular endothelial growth factor activity and suppression of destabilization of hypoxia-inducible factor-1 (Sansal et al, 2004).

In colorectal cancer PTEN is altered through mixed genetic/epigenetic mechanisms (intragenic mutation/epigenetic or 10q23 loss of heterozigosity (LOH)/epigenetic), which lead to the biallelic inactivation of the protein in 20-30% of cases. In addition to PTEN LOH and mutations, *PTEN* promoter hypermethylation is a frequent event in MSI sporadic colorectal cancer and may constitute an important epigenetic mechanism of PTEN inactivation in this setting (Goel et al, 2004). All these alterations lead to the loss of PTEN protein expression and can be altogether analysed by protein expression analyses methods such as western blot (on cells or fresh/frozen tissues) or immunohistochemistry (IHC) on archival fixed tissues. No data have been published about PTEN role in SBA.

Overall, mutations in KRAS, BRAF and PIK3CA genes, as well as the loss of PTEN protein function result in continuous activation of EGFR downstream pathways, regardless of whether the EGFR is activated or pharmacologically blocked.

1.8.9 Targeting EGFR in colorectal cancer: anti-EGFR monoclonal antibodies

Given the important role of EGFR and its downstream pathways in tumorigenesis and disease progression, this receptor has become a relevant and promising target for anti-cancer therapies. In vitro and in vivo studies showed that blocking EGFR and downstream signaling may lead to carcinoma cell growth inhibition, resulting in potential benefits for cancer patients. In colorectal cancer, monoclonal antibodies (MoAb) targeting EGFR, namely cetuximab and panitumumab, have been developed and introduced in clinical practice (Rocha-Lima et al, 2007; Ciardiello and Tortora, 2008). Cetuximab, a human–mouse chimeric IgG1 MoAb, was the first EGFR-targeted agent approved for the treatment of colorectal cancer. Panitumumab, a fully human IgG2 MoAb was recently approved in the US and Europe as third-line treatment of metastatic colorectal cancer (Jonker et al, 2007; Amado et al, 2008; Ciardiello and Tortora, 2008). Cetuximab and panitumumab bind to the extracellular domain of EGFR when it is in the inactive configuration, compete for receptor binding by occluding the ligand-binding region, and thereby block ligand-induced EGFR activation, inducing its internalization and degradation (Ciardiello and Tortora, 2008). Consequently, they block the activation of the EGFR mitogenic signal transduction pathways, and they inhibit therefore tumor cell proliferation, angiogenesis, invasion and metastatic spread by inducing apoptosis. Additionally, anti-EGFR MoAbs, particularly those of the IgG1 subclass, may recruit host immune functions to attack the targeted cancer cell. These functions include antibody-dependent cellular cytotoxicity and, to a lesser extent, complement-mediated cytotoxicity (Kimura et al, 2007; Kurai et al, 2007). Anti-EGFR MoAbs recognize EGFR exclusively and are therefore highly selective for this receptor. Most studies

have been made using cetuximab, but same results are valid also for panitumumab. The ability of cetuximab for blocking the EGFR pathway is supported by preclinical and clinical studies. At preclinical level, it has been demonstrated that cetuximab alone primarily shows cytostatic activity, whereas its combination with other chemotherapeutic agents (such as platinum derived compounds and irinotecan) leads to synergistic antitumoral activity (Fan et al, 1993; Ciardiello et al, 1999; Baselga et al, 2000; Prewett et al, 2003). At clinical level, two phase II trials demonstrated that patients with advanced CRC had a response rate of 11% when cetuximab is administered as single agent therapy, and 23% when combined with irinotecan (Saltz et al, 2004; Cunningham et al, 2004; Chung et al, 2005). Both antibodies have been shown to reduce the risk of tumor progression and to improve overall survival (OS), progression-free survival (PFS) and quality of life of patients with refractory metastatic colorectal cancer (Saltz et al, 2004; Cunningham et al, 2004; Jonker et al, 2007).

1.8.10 Molecular mechanism of response and resistance to EGFR targeted monoclonal antibodies

From early clinical studies conducted mainly in heavily pretreated chemotherapy-refractory patients and also in chemotherapy-naïve patients with advanced colorectal cancer, it became clear that only 10% to 20% of patients with mCRC clinically benefited from anti-EGFR MoAbs (Cunningham et al, 2004; Saltz et al, 2004; Chung et al, 2005). This evidence, together with the side effects and the higher costs of MoAb therapies as compared with standard chemotherapy regimens, underlined the importance of studying the molecular mechanisms of primary resistance to cetuximab or panitumumab.

Initially, it was hypothesized that EGFR targeted agents would be most effective in those tumors overexpressing the EGFR protein (Vogel et al, 2002; Arteaga, 2003). Preclinical studies demonstrated that anti-EGFR agents may have little activity when the level of EGFR expression is below a threshold level (Venook et al, 2005).

Consequently, cetuximab was indicated only for the treatment of patients who have tumors that demonstrated EGFR expression (Cunningham et al, 2004; Saltz et al, 2004; Chung et al, 2005). However, the immunohistochemical expression of EGFR protein turned out to be not a reliable tool for the identification of patients to be treated with EGFR MoAb, due to several reasons: the type of fixative used, the storage time of unstained tissue sections, the type of primary antibody used and the methods of IHC evaluation might generate conflicting data in the EGFR assessment (Atkins et al, 2004; Langner et al, 2004; Kersting et al, 2006). The same was observed with panitumumab-treated patients (Gibson et al, 2006; Siena et al, 2007; Van et al, 2007).

It was next investigated whether alterations of *EGFR* at gene status level might be predictive of anti-EGFR MoAbs efficacy. Several studies showed that *EGFR* gene copy number as detected by Fluorescence In Situ Hybridization (FISH) rather than EGFR protein expression evaluated by IHC, might better predict cetuximab response in advanced colorectal cancer (Moroni et al, 2005; Lièvre et al, 2006, Frattini et al, 2007; Sartore-Bianchi et al, 2007; Cappuzzo et al, 2008). However, a recent contribution demonstrated that FISH seems to be not a reproducible method to evaluate the *EGFR* gene status (Sartore-Bianchi et al, 2012). Therefore, at the moment, *EGFR* gene status is not evaluated as a marker useful for the prediction of anti-EGFR therapies efficacy.

On the contrary, there is a vast consensus for the use of *KRAS* mutations in clinical setting. This type of alteration, leading to the constitutive activation of EGFR downstream pathways, has been clearly demonstrated to be a negative predictor of cetuximab/panitumumab efficacy (Siena and Bardelli, 2010). These data have been included in the Food and Drug Administration (FDA) of the United States, and in the European Medicine Agency (EMA) guidelines. Therefore, at the moment, only patients with *KRAS* wild-type colorectal cancer can be treated with EGFR-targeted therapies.

Since BRAF, PIK3CA and PTEN play a superimposable role with respect to KRAS in the activation of EGFR downstream pathways, it was proposed that also *BRAF* and *PIK3CA* mutations, as well as the loss of PTEN protein expression might identify patients who are resistant to anti-EGFR therapies. Although promising reports opened interesting perspectives confirming this assumption, thus hypothesizing the possibility to identify more than 70% of resistant patients when all the abovementioned markers were investigated simultaneously (Frattini et al, 2007; Di Nicolantonio et al, 2008; Sartore-Bianchi A et al., 2009) (Figure 1.14), other and more recent contributions did not confirmed totally these preliminary data. In particular, it seems that *BRAF* mutations play a prognostic rather than a predictive role in patients treated with EGFR-targeted therapies, that not all *PIK3CA* mutations have the same role (no predictive effect for exon 9 mutations, at odds with those arising in exon 20) and that the loss of PTEN expression should be investigated in the metastatic lesion rather than in the corresponding primary tumor (Loupakis F. et al., 2009; Prenen et al, 2009; De Roock et al, 2010; Custodio et al, 2013). For all these considerations, *BRAF*, *PIK3CA* and PTEN deragulations are not investigated before the administration of EGFR-targeted therapies.

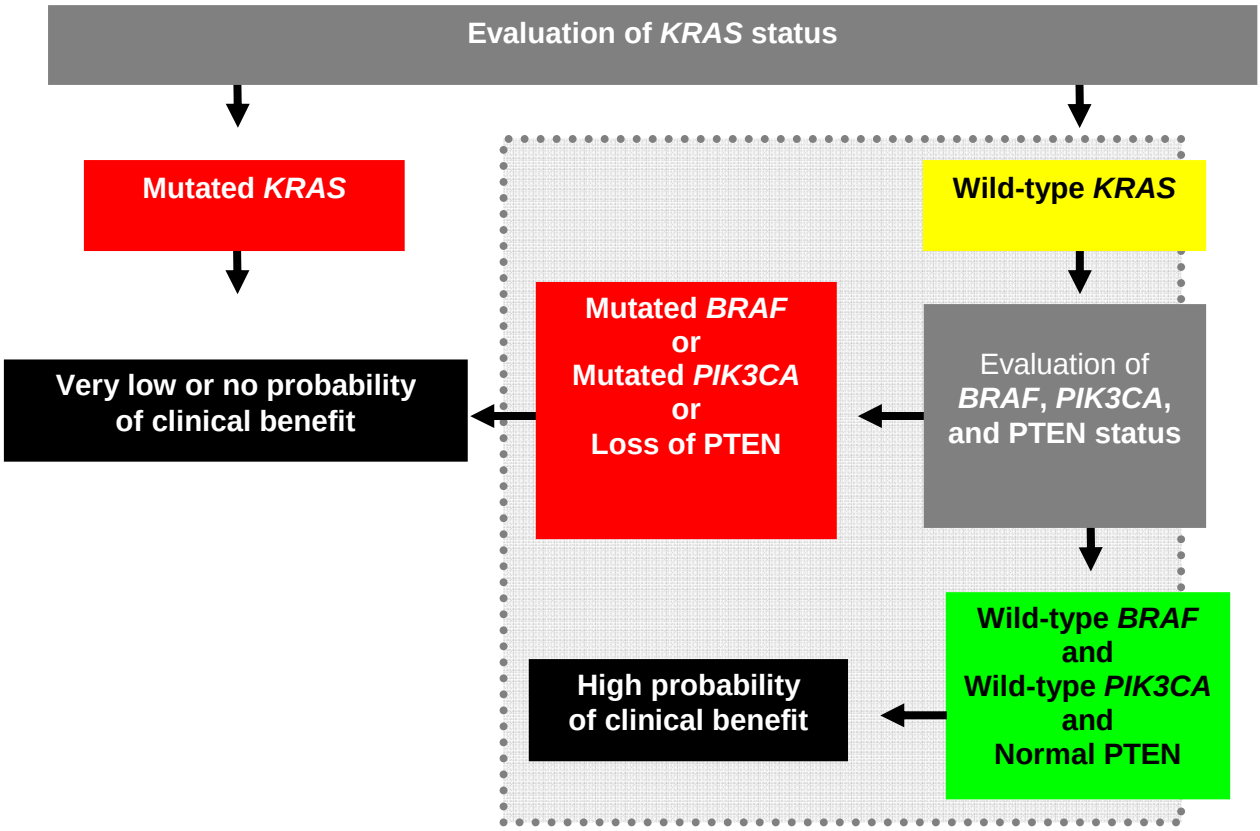

Figure 1.14. Proposed algorithm for a better prediction of EGFR-targeted therapies in metastatic colorectal cancer patients

AIM

Small bowel adenocarcinoma (SBA) is a rare disease presenting high similarity (for histology, epidemiology and risk factors) with colorectal adenocarcinoma. At molecular level, however, little is known for SBA, although it has been proposed that SBA shares the carcinogenetic development with colorectal cancer. For that reason, chemotherapies set up for the cancers of the large intestine have been applied also for SBA.

Primary aim of the present work was the evaluation, in the same cohort, of all the alterations involved in colorectal carcinogenesis, in order to shed light more deeply about the similarity at molecular level between SBA and colorectal cancer. To do this, we will evaluate MSI, β-catenin protein expression, *KRAS* and *TP53* mutations, as well as allelic imbalance of Chromosome 18q.

Recent studies led to the introduction of EGFR-targeted therapies in colorectal cancer. These new compounds showed good benefit, but only in a subgroup of patients. The presence of *KRAS* mutations was demonstrated to be correlated with the absence of efficacy of anti-EGFR drugs. At the same time, a few studies also proposed to investigate *EGFR* gene copy number, *BRAF* and *PIK3CA* mutations, as well as PTEN protein expression, in order to increase the predictive power of the efficacy of EGFR-targeted therapies. Second aim of the present work was therefore to investigate in a cohort of SBA the aforementioned markers involved in the EGFR pathway, in order to verify if the pattern of these alterations could justify the possible introduction of these therapies also in patients affected by SBA.

All the proposed analyses will represent a significant improvement in the knowledge of the molecular characterization of SBA, which will lead to a better treatment of such a severe neoplastic disease.

PATIENTS AND METHODS

3.1 Patients

We recruited 40 patients affected by SBA. Due to the low frequency of occurrence of this type of neoplastic disease, we collected cases from different institutions.

- Sixteen consecutive patients were surgically resected in Canton Tessin and identified in the databases of the Institute of Pathology in Locarno, Switzerland, from 1996 to 2007.
- Five consecutive patients were identified in the Operative Unit of Pathology, Civil Hospital of Legnano (Milan, Italy), from 1997 to 2005.
- Twelve consecutive patients were identified at the Department of Medical Sciences, University of Eastern Piedmont "Amedeo Avogadro" of Novara (Italy), from 2001 to 2008.
- Seven consecutive patients were identified in the databases of Operative Unit of Pathology and Laboratory Medecine of Multimedica (Milan, Italy), from 2003 and 2009.

Patients with tumor of Papilla of Vater were excluded.

3.2 Patient treatment and clinical evaluation

A patient of our cohort was treated with irinotecan but at the end developed a metastatic lesion. Therefore a treatment with cetuximab was proposed.

The patient was treated at Oncology Institute of Southern Switzerland (Bellinzona, Switzerland) with cetuximab in combination with irinotecan with the following scheme:

- cetuximab: loading dose of 400 mg/m^2 over 2 hours, followed by weekly 250 mg/m^2 over 1 hour;
- irinotecan: same dose and schedule used at progression.

Treatment was continued until progressive disease (PD) or toxicity occurred, according to the standard criteria (Therasse et al, 2000) or to specific trial guidelines. Clinical response was assessed every 6 to 8 weeks with radiologic examination (computed tomography or magnetic resonance imaging). The Response Evaluation

Criteria in Solid Tumors (RECIST) (Therasse et al, 2000) were adopted for clinic evaluation, and objective tumor response was classified as partial response (PR), stable disease (SD), or PD.

3.3 Molecular analyses

All the analyses were performed on tumor specimens fixed in 10% buffered formalin and embedded in paraffin (FFPE). FFPE tumor blocks were reviewed for quality and tumor content by analyzing detailed morphology of haematoxylin and eosin stained tissue sections of each blocks. A single representative tumour block from each case, containing at least 70% of neoplastic cells, was selected for immunohistochemical, cytogenetic and molecular analyses. Tumour macrodissection was performed in tumour blocks containing less than 70% of neoplastic cells (to reduce the presence of non-neoplastic tissues) following Van Krieken guidelines (Van Krieken et al, 2008).

3.4 Immunohistochemical analyses

Immunohistochemical analyses were performed on 3-μm thick tissue sections using a Benchmark automatic immunostaining device (Ventana Medical System, Tucson, AZ, USA). According to manufacturer's instructions, specimens were incubated after heat induced antigen retrieval with the specific antibody. More in details, tissue sections were sequentially deparaffinizated with xylene and rehydrated in alcohol solutions and then in distilled water. Subsequently, tissue sections were incubated for 5 minutes with proteinase K, for 5 minutes with peroxidase block solution, for 30 minutes with primary antibody or negative control reagents and for 30 minutes with the secondary goat anti-mouse antibody and horseradish peroxidase molecule linked to a common dextran polymer. At the end, diaminobenzidine (DAB+) substrate chromogen solution was applied for 10 minutes and, after counterstaining with haematoxylin and coverslipping, the sections were kept in the dark at room temperature until the evaluation that was made by using a light microscope. Positive and negative controls were included in each slide run.

β-catenin. β-catenin protein expression analysis was performed using anti-β-catenin monoclonal antibodies (BD Biosciences, San Jose, CA, USA) at 1:50 dilution as previously reported (Frattini et al, 2004). A normal β-catenin expression is considered when tumoral tissues show a cytoplasmic staining similar to that observed in paired healthy mucosa. APC or β-catenin genes alterations lead to an abnormal accumulation of β-catenin protein in the cytoplasm, and a nuclear staining (due to the fact that when the β-catenin protein is too much accumulated in the cytoplasm is able to enter into the nucleus) can also be observed. Therefore, we considered negative cases those showing a cytoplasmic expression of β-catenin protein superimposable with β-catenin expression of healthy mucosa. Then, patients with an abnormally high expression of β-catenin protein were subdivided into 2 groups: those showing overexpression only in the cytoplasm (and named Positive-cytoplasm or Pos Cyt), and those showing also expression in the nucleus (and named Positive-nucleus or Pos Nucl). Healthy tissue (i.e. normal small bowel mucosa) was used as internal control; colorectal adenocarcinoma with a nuclear expression of β-catenin was used as external positive control.

PTEN. PTEN protein expression analysis was performed using the anti-PTEN Ab-4 monoclonal antibodies (Neomarkers, Fremont, CA, USA) at 1:50 dilution as previously reported (Frattini et al, 2005; Saal et al, 2005; Frattini et al, 2007). PTEN staining intensity scores for invasive tumor and non-neoplastic cells were recorded as described in the literature (Saal et al, 2005) and on the basis of our experience (Frattini et al, 2007). PTEN protein expression was mainly detected at the cytoplasmic level, while very few cases also showed nuclear positivity. We considered PTEN negative tumours those showing a striong reduction or absence of immunostaining in at least 50% of cells, as compared with the internal control (i.e., vascular endothelial cells and nerves). Healthy tissue (i.e. normal colon mucosa) was used as internal positive control; normal endometrium was used as external positive control.

3.5 Fluorescent in situ hybridization (FISH)

EGFR gene status evaluation was performed on 3-µm thick tissue sections that were treated using Paraffin Pretreatment kit II (Abbott Molecular, AG Baar, Switzerland) according to manufacturer's instructions. Dual-colour FISH assay was performed using LSI *EGFR/CEP7* probes (Abbott Molecular). The LSI *EGFR* probe is labelled in SpectrumOrange and covers an approximately 300 kb region that contains the entire *EGFR* gene at 7p12 (red signals). The *CEP7* probe, labelled in SpectrumGreen (green signals), hybridises to the alpha satellite DNA located at the centromere of Chromosome 7 (7p11.1– q11.1) (Figure 3.1).

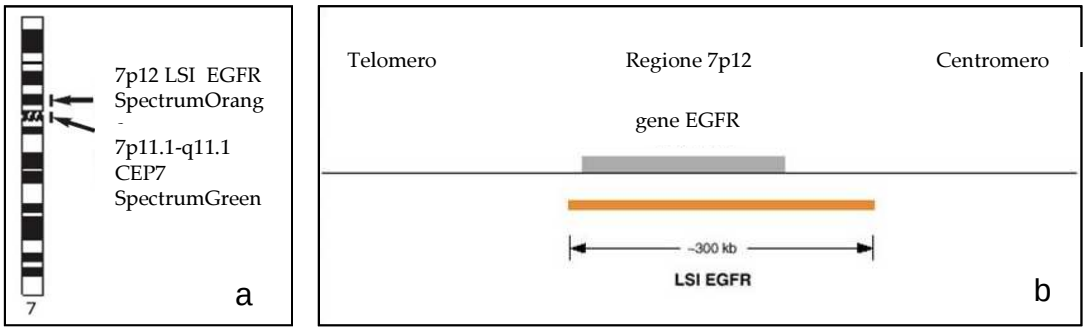

Figure 3.1. Visual representation of LSI *EGFR/CEP7* dual colour probe; **a**: Probes position and types with respect to the entire Chromosome 7; **b**: Position of *EGFR* probe with respect to *EGFR* gene (www.abbottmolecular.com).

Target sections and probes were co-denatured at 75°C for 5 min and allowed to hybridize overnight at 37°C. A post-hybridisation stringency wash was carried out in a water bath at 72°C for 5 min. After washing twice and drying at room temperature for 10 min, slides were mounted with 406-diamidino-2-phenylindole (DAPI II; Vysis). Fluorescent in situ hybridization signals were evaluated with a fluorescent automated microscope (Zeiss Axioplan 2 Imaging, Zeiss, Oberkochen, Germany) equipped with single and triple band pass filters. Image for documentation were captured using an AxioCam camera (Zeiss Axiocam MRm) and processed using the AxioVision system (Zeiss). Patients were classified using descriptive criteria, taking

into account the abnormalities revealed and the percentage of cells involved (Martin et al, 2009).

To overcome the problem of tissue heterogeneity, we evaluated 10 different tumour areas and at least 10 representative nuclei from each area. Overall, a total of 100 cells for each patient were scored. For cases in which only a biopsy was available, we evaluated all the analysable nuclei.

A normal cell is characterized by 2 red signals and 2 green signals (Figure 3.2a), or by 4 red signals and 2 green signals during DNA replication (Figure 3.2b).

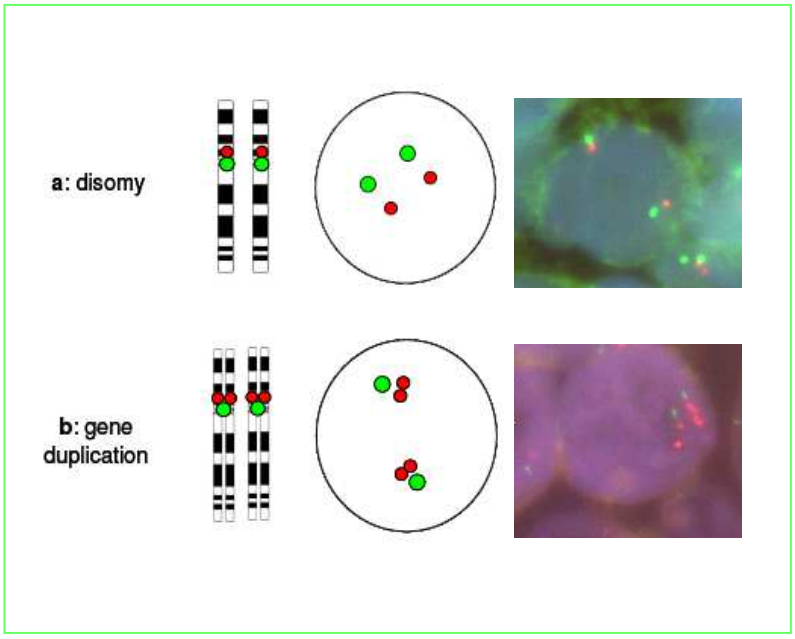

Figure 3.2. Schematic representation of *EGFR* FISH on chromosomes and in cells. Green signals correspond to the centromere of Chromosome 7, red signals to *EGFR* gene. **a**: normal nucleus; **b**: normal nucleus with DNA replication.

In all the other situations there is an abnormal *EGFR* gene status, depending on different mechanisms:

1) alterations linked to the number of Chromosomes 7;
2) alterations of *EGFR* gene itself;

In the first category we can identify the following situations:

- loss of Chromosome 7 (also named monosomy) (Figure 3.3a);
- low polysomy (3 or 4 balanced copies of red and green signals) (Figure 3.3b);

- high polysomy (more than 4 balanced copies of red and green signals) (Figure 3.3c).

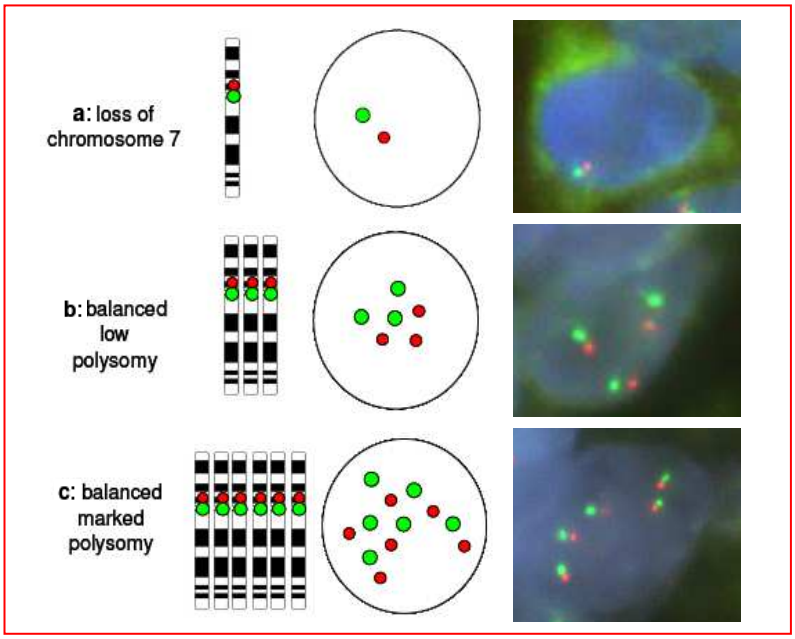

Figure 3.3. Schematic representation of EGFR alterations linked to the number of Chromosome 7). **a**: loss of Chromosome 7; **b**: low polysomy (for example, 3 copies of the Chromosome 7); **c**: high polysomy (more than 4 copies, in the example there are 6 copies, of the Chromosome 7).

EGFR alterations due to a problem of *EGFR* gene itself are caused by gene amplification. In general, in this situation, the number of red signals is higher than that of green signals, therefore the ratio (R) between red and green signals is more than 2. In this category, we can identify 3 different situations, although each of them leads to an abnormally high number of *EGFR* gene:

- low level of gene amplification (when 2<R<5) (Figure 3.4a);
- high level of amplification HSR-type (Homogenously-Stained Regions), when R>5 and red signals are in clusters (Figura 3.4b);
- high level of amplification DM-type (Double Minutes), when R>5 and red signals are dispersed in the nucleus and correspond to extra-chromosomal sequences without centromere (Figura 3.4c).

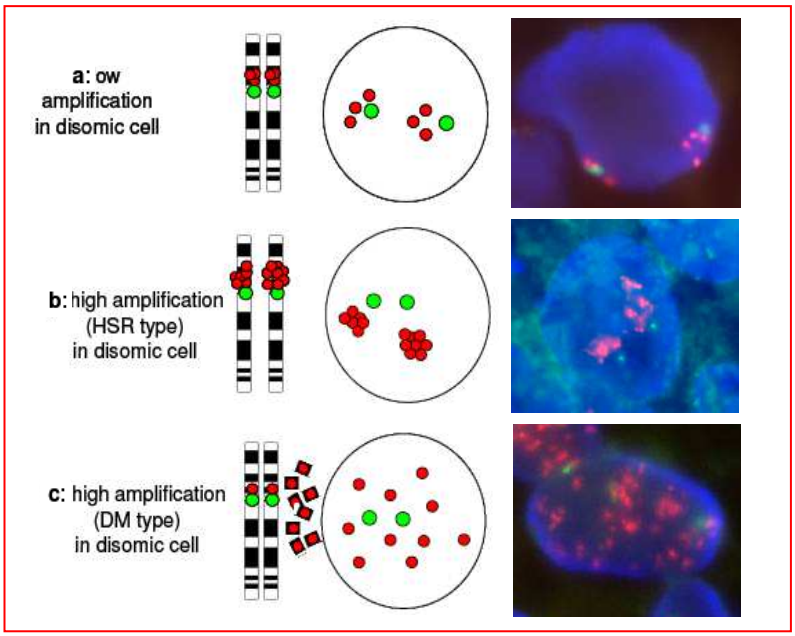

Figure 3.4. Schematic representation of *EGFR* gene amplificfation (R>2). **a:** low level of gene amplification (R<5); **b:** high level of amplification HSR-type (Homogenously-Stained Regions); c: high level of amplification DM-type (Double Minutes).

On the basis of criteria published for colorectal cancer (Martin et al, 2009; Varella Garcia et al, 2009), cases showing only 1 Chromosome 7 were classified as *EGFR* loss. When we observed 2 Chromosomes 7 in more than 60% of cells, the tumor was classified as disomic. Tumour samples with an aberrant number of Chromosomes 7, defined as ≥ 3 copies in at least 40% of cells, were classified as polysomic and: low polysomic in the presence of 3 copies of Chromosomes 7, tetrasomic in the presence of 4 copies of Chromosomes 7, highly polysomic in the presence of more than 4 copies of Chromosomes 7. Specimens with a R>2 between *EGFR* gene and Chromosomes 7 centromere signals in at least 10% of cells were classified as carrying *EGFR* gene amplification. As patients carrying either at least a tetrasomic profile or gene amplification show a significant gain of *EGFR* gene, we grouped them into a class named copy number gain (CNG), according to the criteria described in the literature with slight modifications (Moroni et al, 2005): this group is also considered to be FISH positive (FISH+). Loss of Chromosomes 7, disomy as well as low polysomy are cumulatively considered as FISH negative (FISH-). Table 3.1 includes all classifications we applied.

Categories	Anomaly	Number of cells	Status
Loss (L)	1 copy of Chromosome 7	> 50%	FISH-
Disomy (D)	2 copies of Chromosome 7	> 60%	FISH-
Low Polysomy (LP)	3 or 4 copies of Chromosome 7	> 40%	FISH- FISH+(*)
High polysomy (HP)	more than 4 copies of Chromosome 7	> 40%	FISH+
Gene Amplification (A)	R>2	> 10%	FISH+

Table 3.1. Interpretation criteria for *EGFR* FISH (Martin et al, 2009; Varella Garcia et al, 2009). Legend: R: ratio between gene and centromere signals (red and green signals, respectively), (*) only if tetrasomy.

3.6 Mutational analysis by direct sequencing

Genomic DNA was extracted using the QIAamp Mini kit (Qiagen, Chatsworth, CA, USA) according to manufacturer's instructions.

KRAS (exons 2 and 3), *BRAF* (exon 15), *PIK3CA* (exons 9 and 20) and *TP53* (exons 4-10) gene mutations were detected by direct sequencing on genomic DNA as already reported (Frattini et al, 2004; Frattini et al, 2005; Di Nicolantonio et al, 2008). *KRAS* exon 2 includes codons 12 and 13, *KRAS* exon 3 includes codon 61, *BRAF* exon 15 includes codon 600, *PIK3CA* exon 9 includes codons 542 and 545 and *PIK3CA* exon 20 includes codon 1047. All these codons represent sites where the large majority of oncogenic mutations occur (Davies et al, 2002; Frattini et al, 2004; Samuels et al, 2004). As for *TP53* gene, there are not specific hotspots (although a higher percentage of alterations is observed in codons 175, 245, 248 and 273), and the mutations can occur in all the nucleotides between exon 4 and exon 10: therefore we amplified all these exons. The nucleotide sequence corresponding to every exon was amplified from tumour-extracted genomic DNA by Polimerase Chain Reaction (PCR), purified (Microcon YM-50, Millipore, Billerica, MA, USA) and directly sequenced. The list of primers used for mutational analyses is reported in Table 3.2. All samples were subjected to automated sequencing by ABI PRISM 3130 (Applied Biosystems, Foster City, CA, USA). All mutated cases were confirmed at least twice

starting from independent PCR reactions. In each case, the detected mutation was confirmed in the sequence as sense and antisense strands.

Gene	Exon	Forward Primer	Reverse Primer	Annealing Temperature (°C)
K-Ras	2	5′-TGGTGGAGTATTTGATAGTGTA-3′	5′-CATGAAAATGGTCAGAGAA-3′	55
K-Ras	3	5′-GGTGCACTGTAATAATCCAGA-3′	5′-TGATTTAGTATTATTTATGGC-3′	49
BRAF	15	5′-TCATAATGCTTGCTCTGATAGGA-3′	5′-GGCCAAAAATTTAATCAGTGGA-3′	52
PIK3CA	9	5′-GGGAAAAATATGACAAAGAAAGC-3′	5′-CTGAGATCAGCCAAATTCAGTT-3′	56
PIK3CA	20	5′-CTCAATGATGCTTGGCTCTG-3′	5′-TGGAATCCAGAGTGAGCTTTC-3′	55
TP53	4.1	5′-GAGGACCTGGTCCTCTGACT-3′	5′-AAGGGACAGAAGATGACAGG-3′	60
TP53	4.2	5′-AGAGGCTGCTCCCCGCGTGG-3′	5′-ATACGGCCAGGCATTGAAGT-3′	60
TP53	5	5′-TTCAACTCTGTCTCCTTCCT-3′	5′-CAGCCCTGTCGTCTCTCCAG-3′	62
TP53	6	5′-GCCTCTGATTCCTCACTGAT-3′	5′-TTAACCCCTCCTCCCAGAGA-3′	62
TP53	7	5′-AGGCGCACTGGCCTCATCTT-3′	5′-TGTGCAGGGTGGCAAGTGGC-3′	64
TP53	8	5′-TTCCTTACTGCCTCTTTGCTT-3′	5′-AAGTGAATCTGAGGCATAAC-3′	56
TP53	9	5′-AGCAAGCAGGACAAGAAGCG-3′	5′-ACTTGATAAGAGGTCCCAAG-3′	58
TP53	10	5′-TTTTAACTCAGGTACTGTGT-3′	5′-CTTTCCAACCTAGGAAGGCA-3′	58

Table 3.2. Genes analyzed, primers sequences and annealing temperatures.

3.7 MSI

The status of MSI was assessed by the analysis of the microsatellite loci included in the panel of Bethesda (*BAT25*, *BAT26*, *D2S123*, *D5S346*, and *D17S250*), as reported in the literature (Frattini et al, 2004). The list of primers used for MSI analysis is reported in Table 3.3. MSI was confirmed by the presence of additional peak(s) in tumor sample compared with the pattern of the normal paired tissue. MSI was defined as being present when more than 30% of investigated loci showed instability.

Exon	Forward Primer	Reverse Primer	Annealing Temperature (°C)
BAT25	5′-TCGCCTCCAAGAATGTAAGT-3′	5′-TCTGCATTTTAACTATGGCTC-3′	52
BAT26	5′-TGACTACTTTTGACTTCAGCC-3′	5′-AACCATTCAACATTTTTAACCC-3′	52
D2S123	5′-AAACAGGATGCCTGCCTTTA-3′	5′-GGACTTTCCACCTATGGGAC-3′	52
D5S346	5′-ACTCACTCTAGTGATAAATCGGG-3′	5′-AGCAGATAAGACAGTATTACTAGTT-3′	52
D17S250	5′-GGAAGAATCAAATAGACAAT-3′	5′-GCTGGCCATATATATATTTAAACC-3′	52

Table 3.3. Loci analyzed, primers sequences and annealing temperatures. Forward primers are labeled with 6-FAM at 5′-end

3.8 Allelic imbalance of Chromosome 18q

Several tumor suppressor genes are located on Chromosome 18. Of these, 3 genes play a relevant role in CRC: *DCC, SMAD2* (previously named *JV18* or *MADR2*) e *SMAD4* (previously named *DPC4* or *MADR4*), located on the long arm of Chromosome 18 (i.e: q portion of the chromosome). To evaluate if Chromosome 18q region is lost, thus meaning that these tumor suppressor genes are absent and therefore cannot exert their biologic activity, it is useful to investigate the allelic imbalance, also named as loss-of-heterozygosity.

On the basis of our previous experience (Frattini et al, 2004), we included in our analysis the following loci: *D18S64, D18S484, D18S474, D18S1110, D18S1161, D18S68, D18S1102* (see Figure 3.5 for the location of these loci with respect to DCC, SMAD2 and SMAD4 genes).

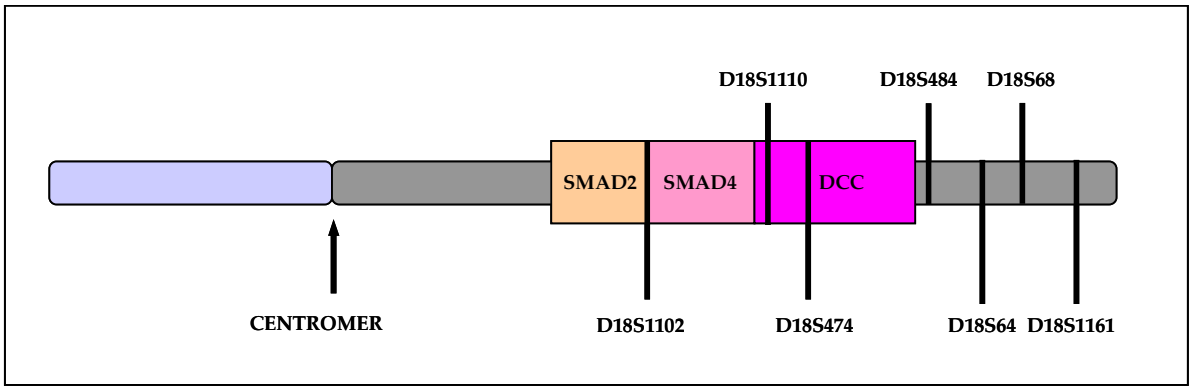

Figure 3.5. Loci position with respect to the centromere of Chromosome 18q and with respect to *DCC, SMAD2* and *SMAD4* genes.

These loci were analyzed according to the protocol already published (Frattini et al, 2004). The list of primers used for allelic imbalance of Chromosome 18q is reported in Table 3.4.

Exon	Forward Primer	Reverse Primer	Annealing Temperature (°C)
D18S64	5′-ATACTGGTGGTGGTTATACAACAT-3′	5′-AAATCAGGAAATCGGCA-3′	52
D18S484	5′-TGTAGCATTTTTAAGACAGTAAAG-3′	5′-ACATATTCCTTGCTTTGTCA-3′	52
D18S474	5′-TGGGGTGTTTACCAGCATC-3′	5′-TGGCTTTCAATGTCAGAAGG-3′	52
D18S1110	5′-TGACCTTGGCTACCTTGC-3′	5′-TCGAAAGCCTTAAACTCTGA-3′	52
D18S1161	5′-GTCCGTCCAACGTCCAA-3′	5′-GGAGAGCCACACCTATCCTG-3′	52
D18S68	5′-ATGGGAGACGTAATACACCC-3′	5′-ATGCTGCTGGTCTGAGG-3′	52
D18S1102	5′-TTTCAGGATTTTGGAGCC-3′	5′-GGAATGACTGCGTCTGTG-3′	52

Table 3.4. Loci analyzed, primers sequences and annealing temperatures. Forward primers are labeled with 6-FAM at 5′-end.

The analysis requires the availability of healthy tissue. If in the healthy tissue sample the 2 alleles have the same size, the patient is homozygous for that locus and cannot be evaluated for the analysis of allelic imbalance (the locus is classified as "not informative" (NI)).

If in the tumoral tissue there is the presence of additional peak(s) compared with the normal paired tissue, the locus is considered "instable" (or MSI), and again it is considered NI for the analysis of allelic imbalance.

If in both normal and tumoral tissue there are 2 alleles with different sizes, the patient is heterozygous for that locus and the allelic imbalance can be ascertained, using the following formula:

$$R = \frac{N1}{N2} \times \frac{T2}{T1}$$

where

N1 = height of lower dimension peak of healthy tissue

N2 = height of higher dimension peak of healthy tissue

T1 = height of lower dimension peak of tumoral tissue

T2 = height of higher dimension peak of tumoral tissue.

As cut-off, on the basis of the literature (Frattini et al, 2004), we used the value of 30%. Therefore, if R is between 0.7 and 1.3, the locus is normal, not lost. If $R \leq 0.70$ or

$R \geq 1.3$, there is allelic imbalance, that means that 1 allele is lost. A patient is classified as carrying the loss of the Chromosome 18q region when at least 30% of informative loci have $R \leq 0.70$ o $R \geq 1.3$.

3.9 Statistical analyses

For all the statistical correlations, we used the Fisher's exact test because it is appropriate for the analysis of small number of cases and for zero values. The level of significance was set at $p=0.05$.

RESULTS

4.1 Patients cohort

Between 1996 and 2009, 40 patients affected by SBA were identified in the databases of Institute of Pathology of Locarno (Canton Tessin, Southern Switzerland) and in those of three institutions of Northern Italy (Departments of Pathology of Legnano, Multimedica in Milan, and University of Novara).

The age at diagnosis was between 41 and 87 years, with a mean age of 66.5 years. Men and women were equally distributed (20 cases for both sexes). Tumor location included duodenum (35% of cases), jejunum (25% of cases) and ileum (40% of cases).

Tumor stage was available in 36 cases, because for 4 patients (PT = 9, 12, 15 and 16) (Table 4.1) only a biopsy was available. One case (2.5%) was classified as pT1, 2 cases (5%) as pT2. Therefore, the vast majority of cases were classified at higher stage, as pT3 in 24 cases (60%) and as pT4 in 9 cases (22.5%).

The majority of cases was classified as G2 (26 cases, 65%), with 9 cases (22.5%) as G3 and only 3 cases (7.5%) as well differentiated (G1). Two patients (PT = 30 and 31, Table 4.1) were G2 with few portions classified as G3 (5%), but were grouped with G3 (overall G3: 30% of cases).

In 4 patients the lymph nodal involvement was not available (Nx). Of the remaining 36 cases, 15 patients displayed lymph nodal metastasis (pN1).

As far as metastasization of other organs is concerned, the information was not available in 5 cases (pMx). Of the remaining 35 cases, 5 patients displayed a distant metastasis at the time of diagnosis of the primary tumor (pM1).

Of the whole cohort, only 1 case was excluded (PT = 12, Table 4.1) because the DNA was not analyzable for both direct sequencing and FISH experiments. We decided not to analyze this case also at immunohistochemical level. Therefore, the analyses was accomplished on 39 patients.

PT id	Sex	Age at diagnosis	Tumor location	TNM stage (grade)
1	M	74	Jejunum	pT3 pN1 (G3)
2	M	78	Duodenum	pT3 pN0 (G3)
3	M	57	Ileum	pT3 pN0 pM1 (G2)
4	M	66	Ileum	pT4 pN1 (G3)
5	M	59	Duodenum	pT3 pN1 (G2)
6	F	72	Duodenum	pT4 pN0 (G2)
7	M	87	Duodenum	pT1 pNx (G2)
8	F	74	Duodenum	pT4 pN1 (G3)
9	M	41	Duodenum	(G2)
10	F	76	Ileum	pT4 pN1 (G3)
11	M	78	Jejunum	pT3 pN1 (G2)
12	M	66	Duodenum	(G1)
13	M	65	Duodenum	pT3 pN1(G3)
14	F	61	Duodenum	pT4 pN1 (G2)
15	F	87	Duodenum	(G2)
16	F	81	Duodenum	(G3)
17	M	67	Jejunum	pT3 pN0 (G2)
18	F	59	Jejunum	pT3 pN0 (G2)
19	F	80	Ileum	pT4 pN0 pM1 (G2)
20	F	42	Ileum	pT4 pNx pM1 (G2)
21	M	77	Ileum	pT3 pN1 (G2)
22	M	73	Duodenum	pT3 pN0 (G2)
23	M	71	Ileum	pT3 pN0 (G2)
24	F	63	Ileum	pT3 pN0 pMx (G1)
25	M	73	Duodenum	pT3 pN0 (G2)
26	M	62	Ileum	pT4 pN1 (G1)
27	F	68	Ileum	pT3 pNx pMx (G2)
28	M	62	Ileum	pT3 pN0 pMx (G2)
29	M	67	Duodenum	pT4 pN1 pM1 (G2)
30	F	66	Jejunum	pT3 pN1 pM1 (G2-G3)
31	F	68	Jejunum	pT3 pN1 pMx (G2-G3)
32	F	80	Jejunum	pT3 pN0 (G2)
33	M	48	Jejunum	pT3 pNx pMx (G3)
34	F	69	Jejunum	pT3 pN0 (G2)
35	M	60	Jejunum	pT2 pN0 (G2)
36	F	70	Ileum	pT2 pN0 (G2)
37	F	69	Ileum	pT3 pN2 (G2)
38	F	83	Ileum	pT3 pN0 (G2)
39	F	63	Ileum	pT3 pN1 pM1 (G3)
40	F	73	Ileum	pT3 pN1 (G2)

Table 4.1. Clinico-pathological features of patients affected by SBA and surgically removed in Canton Tessin and in Italy (Legnano, Milan and Novara) from 1996 to 2009.
Legend: F: female; M: male; PT id: patient identification; TNM: Tumor, Node, Metastasis system.

4.2 β-Catenin protein expression

Due to lack of material, 1 case was not evaluable (PT = 9).

Eleven patients displayed a negative expression of β-catenin (an example is reported in Figure 4.1), whereas 9 patients (PT = 2, 10, 20, 23, 24, 26, 27, 32 and 36) were characterized by a strong protein expression, with a marked immunodecoration not only in the cytoplasm but also in the nucleus (Figure 4.2). The remaining 18 patients showed β-catenin overexpression, higher than in the healthy mucosa, but only at cytoplasmic level (Table 4.2).

PT id	β-Catenin
1	Pos (cyt)
2	Pos (cyt + nucl)
3	Pos (cyt)
4	Neg
5	Neg
6	Pos (cyt)
7	Pos (cyt)
8	Neg
10	Pos (cyt + nucl)
11	Pos (cyt)
13	Neg
14	Neg
15	Neg
16	Pos (cyt)
17	Neg
18	Pos (cyt)
19	Pos (cyt)
20	Pos (cyt + nucl)
21	Pos (cyt)

PT id	β-Catenin
22	Pos (cyt)
23	Pos (cyt + nucl)
24	Pos (cyt + nucl)
25	Pos (cyt)
26	Pos (cyt + nucl)
27	Pos (cyt + nucl)
28	Pos (cyt)
29	Pos (cyt)
30	Neg
31	Pos (cyt)
32	Pos (cyt + nucl)
33	Neg
34	Neg
35	Pos (cyt)
36	Pos (cyt + nucl)
37	Pos (cyt)
38	Pos (cyt)
39	Neg
40	Pos (cyt)

Table 4.2. β-catenin protein expression in evaluable cases, by immunohistochemistry. Cases with a strong nuclear expression are labeled in red, those with only cytoplasmic overexpression in green.
Legend: cyt = cytoplasmic expression; neg = negative expression; nucl = nuclear expression; pos = positive expression; PT id= patient identification.

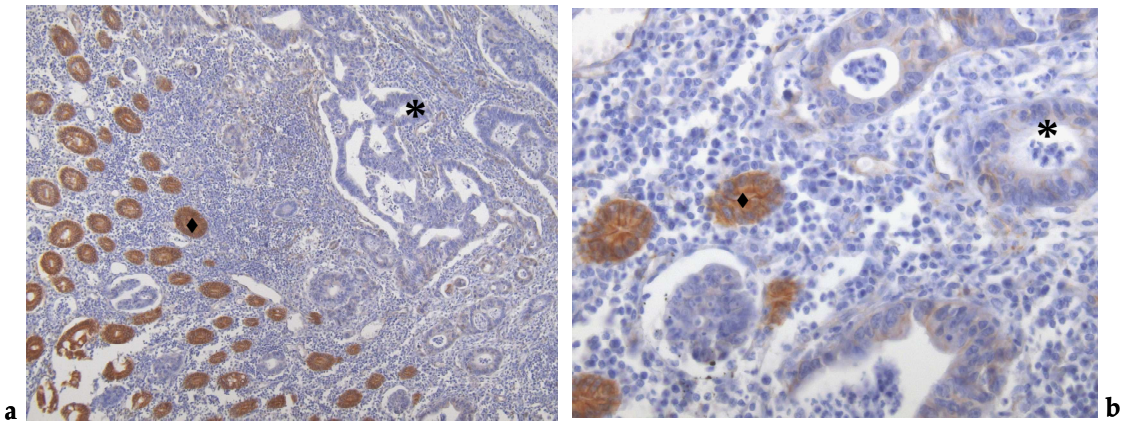

Figure 4.1. a: example of negative expression of β-catenin in SBA. **b:** higher magnification of the same region. ♦: normal tissue; *: adenocarcinoma.

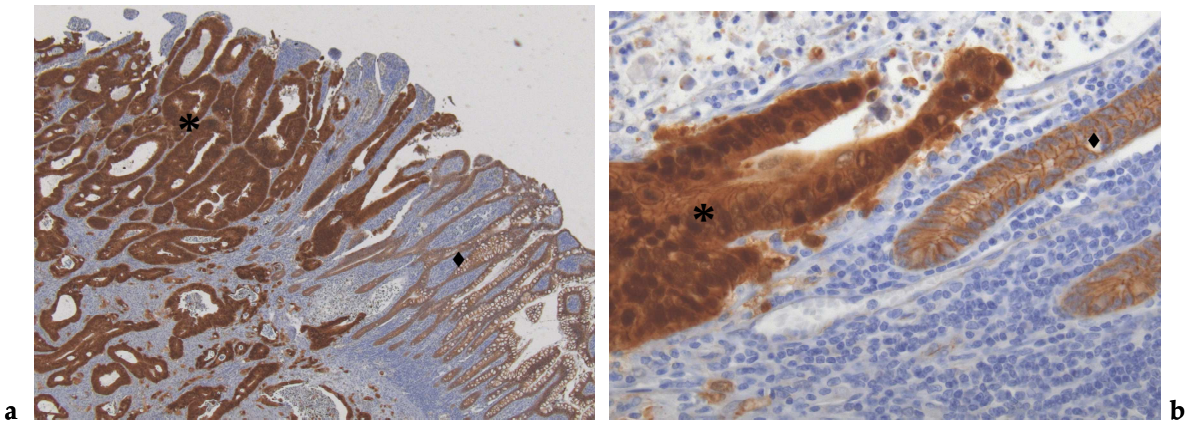

Figure 4.2. a: example of strong expression (cytoplasmic and nuclear) of β-catenin in SBA. **b:** higher magnification of the same region. ♦: normal tissue; *: adenocarcinoma.

4.3 Microsatellite instability analysis

This analysis foresees the amplification of genomic DNA from both tumoral and non tumoral portions. For patients with only a biopsy available, normal tissue was obtained from another resection, or by microdissection on the same section used for DNA extraction of the tumoral portion. Therefore, the analysis of all the loci included in the panel of Bethesda was allowed in all the cases.

Due to lack of material, 1 case was not investigated (PT = 9).

Twenty-nine cases were characterized by microsatellite stability (MSS). Nine patients, on the contrary, were classified as microsatellite instable (MSI).

Overall, in our cohort of SBA we found 23.6% of cases with MSI feature.

PT id	Microsatellite status
1	MSI
2	MSS
3	MSS
4	MSS
5	MSI
6	MSS
7	MSS
8	MSS
10	MSS
11	MSS
13	MSI
14	MSS
15	MSI
16	MSS
17	MSI
18	MSS
19	MSS
20	MSS
21	MSS

PT id	Microsatellite status
22	MSS
23	MSS
24	MSS
25	MSS
26	MSS
27	MSS
28	MSI
29	MSS
30	MSI
31	MSS
32	MSS
33	MSS
34	MSI
35	MSS
36	MSS
37	MSS
38	MSS
39	MSI
40	MSS

Table 4.3. Microsatellite instability analysis in SBA evaluable cases. Microsatellite instable cases are reported in red.
Legend: MSI = microsatellite instability; MSS = microsatellite stability; PT id= patient identification.

4.4 *KRAS* gene mutations

KRAS mutational status, performed by direct sequencing of both exons 2 and 3, including codons 12, 13 and 61, was performed in 39 cases (Table 4.4). In 22 cases a wild-type sequence in both exons was observed. In 17 cases a point mutation was identified. More in details, 16 mutations occurred in exon 2 and 1 mutation in exon 3. In exon 2, all the mutations were discovered in codon 12: 6 mutations were represented by G12D, 3 by G12S, 3 by G12V, 2 by G12C, 1 by G12A e another 1 by G12R. As far as exon 3 is concerned, the mutation was identified at codon 61, represented by Q61H change (Table 4.4).

Cumulatively, *KRAS* mutations are observed in 43.6% of our cohort of SBA.

PT id	*KRAS*
1	G12S
2	WT
3	WT
4	WT
5	G12S
6	G12D
7	WT
8	WT
9	WT
10	G12C
11	G12A
13	WT
14	G12R
15	G12V
16	WT
17	WT
18	WT
19	G12D
20	G12V
21	G12C

PT id	*KRAS*
22	WT
23	WT
24	WT
25	WT
26	WT
27	G12V
28	Q61H
29	WT
30	WT
31	WT
32	WT
33	WT
34	WT
35	G12D
36	G12S
37	WT
38	G12D
39	G12D
40	G12D

Table 4.4. Mutational status of evaluable cases of *KRAS* gene (exons 2 and 3), by direct sequencing. Cases carrying a mutation are reported in red
Legend: PT id= patient identification; WT = wild-type. In the mutant cases, numbers identify the altered codon, the letter in the left of the number represents the wild-type amino acid sequence, the letter in the right of the number represents the altered amino acid.

4.5 Chromosome 18q analysis

This analysis foresees the amplification of genomic DNA from both tumoral and non tumoral portions. For patients with only a biopsy available, normal tissue was obtained from another resection, or by microdissection on the same section used for DNA extraction of the tumoral portion.

Three patients were excluded due to lack of material (PT = 9, 16 and 29). Two cases were considered to be not evaluable due to the bad quality of genomic DNA, at least for the analysis of the long arm of Chromosome 18 (PT = 25 and 37). In addition, the 9 patients showing MSI at the loci included in the panel of Bethesda, were also characterized by microsatellite instability in the loci of Chromosome 18q, and for that reason were excluded (Table 4.5).

In conclusion, data useful for the characterization of the allelic imbalance of the long arm of Chromosome 18 were obtained in 25 cases. Of these, 6 patients did not show any loss of the Chromosome 18q region, whereas 19 experienced loss-of-heterozygosity.

Therefore, the region including tumor suppressor genes located on Chromosome 18q was lost in 76% of SBA.

PT id	Chromosome 18q
1	MSI
2	NL
3	LOH
4	NL
5	MSI
6	NL
7	LOH
8	LOH
10	LOH
11	LOH
13	MSI
14	NL
15	MSI
17	MSI
18	LOH
19	NL
20	LOH

PT id	Chromosome 18q
21	NL
22	LOH
23	LOH
24	LOH
26	LOH
27	LOH
28	MSI
30	MSI
31	LOH
32	LOH
33	LOH
34	MSI
35	LOH
36	LOH
38	LOH
39	MSI
40	LOH

Table 4.5. Chromosome 18q allelic imbalance in SBA evaluable cases. Cases with loss-of-heterozygosity are reported in red.
Legend: LOH = loss-of-heterozygosity; MSI = microsatellite instability; NL = non loss; PT id= patient identification.

4.6 *TP53* gene mutations

The mutational analysis of *TP53* gene was successful in 38 cases, 1 patient (PT = 9) was excluded due to lack of material.

In 27 patients we did not observe any mutation in all the investigated exons, while 11 patients were characterized by at least one alteration. More in details, 9 patients displayed 1 mutation, and 2 patients showed 2 distinct mutations (Table 4.6).

The mutations identified in patients characterized by 1 mutation were as follows:

- Exon 4.2: deletion of 3 nucleotides determining the loss of codon 96 and leading therefore to a shorter protein (PT = 30, Table 4.6);
- Exon 5: missense point mutation leading to the R175H amino acid change (PT = 10, Table 4.6) and 2 frameshift alterations (PT = 20 and 33, Table 4.6). The frameshift of PT = 20 could not be characterized because the nucleotide deletion was too close to the primer sequence. The frameshift alteration of PT = 33 was represented by the deletion of nucleotide C in position 459 (located in codon 153), leading to an abnormal stop codon at codon 169;
- Exon 6: non-sense point mutation leading to the stop codon in position 213 (PT = 19, Table 4.6);
- Exon 7: 3 missense point mutations, leading to C242Y aminoacid change in 1 case (PT = 18, Table 4.6), and to R248W change in 2 cases (PT = 24 and 31, Table 4.6);
- Exon 8: missense point mutation leading to R282W aminoacid change (PT = 32, Table 4.6);

For patients with 2 distinct mutations, we observed the following changes: PT = 2 (Table 4.6) was characterized by the presence of missense point mutations leading to R213Q (exon 5) and G245V (exon 6) amino acid changes; PT = 17 (Table 4.6) was characterized by missense point mutations leading to R181C (exon 5) and R273H (exon 8) amino acid changes.

No alterations were identified in exons 9 and 10.

Cumulatively, 29% of our cohort of SBA displayed at least a *TP53* mutation.

PT id	*TP53*
1	WT
2	R213Q/G245V
3	WT
4	WT
5	WT
6	WT
7	WT
8	WT
10	R175H
11	WT
13	WT
14	WT
15	WT
16	WT
17	R181C/R273H
18	C242Y
19	R213STOP
20	FRAMESHIFT
21	WT

PT id	*TP53*
22	WT
23	WT
24	R248W
25	WT
26	WT
27	WT
28	WT
29	WT
30	FRAMESHIFT
31	R248W
32	R282W
33	FRAMESHIFT
34	WT
35	WT
36	WT
37	WT
38	WT
39	WT
40	WT

Table 4.6. Mutational status of SBA evaluable cases of *TP53* gene, by direct sequencing. Cases carrying a mutation are reported in red
Legend: PT id = patient identification; WT = wild-type. In the mutant cases, numbers identify the altered codon, the letter in the left of the number represents the wild-type amino acid sequence, the letter in the right of the number represents the altered amino acid.

4.7 *EGFR* gene status.

Six patients (PT = casi 7, 13, 23, 25, 29 and 37, Table 4.7) were not evaluable due to fixation artifacts or to lack of material.

Four patients (PT = 1, 9, 17 and 30, Table 4.7) showed a disomic pattern (2n) in more than 60% of cells; 2 patients (PT = 15 and 38, Table 4.7) were characterized by LP, that means trisomy and/or tetrasomy, in at least 40% of cells; 8 patients showed disomy in more than 60% of cells and LP in remaining cells (2n+LP); 13 cases demonstrated HP in at least 40% of cells; 4 patients (PT = 18, 27, 32 and 34, Table 4.7) were characterized by LP with prevalence of tetrasomy (4n) in at least 40% of cells), 1 patient (PT = 20, Table 4.7) showed LP in at least 40% of cells and a little percentage of cells with gene amplification (LP+a); and 1 cases (PT = 33, Table 4.7) showed gene amplification (a).

Following the criteria described in 3.4.1 section, the 2n, LP (trisomy) and 2n+LP (trisomy) groups were classified as FISH negative (FISH-) (Figure 4.3, Table 4.7) while HP, LP+a e a groups as FISH positive (FISH+) (Figure 4.4, Table 4.7).

Overall, 19 out of 33 evaluable cases were considered as FISH+ (57.5%).

PT id	Categories	Status
1	2n	FISH -
2	HP	FISH +
3	HP	FISH +
4	2n+LP (3n)	FISH -
5	2n+LP (3n)	FISH -
6	2n+LP (3n)	FISH -
8	HP	FISH +
9	2n	FISH -
10	2n+LP (3n)	FISH -
11	HP	FISH +
13	NV	NV
14	HP	FISH +
15	LP (3n)	FISH -
16	HP	FISH +
17	2n	FISH -
18	4n	FISH +
19	HP	FISH +
20	LP+a	FISH +

PT id	Categories	status
21	2n+LP (3n)	FISH -
22	HP	FISH +
24	HP	FISH +
25	NV	NV
26	HP	FISH +
27	4n	FISH +
28	2n+LP (3n)	FISH -
30	2n	FISH -
31	HP	FISH +
32	4n	FISH +
33	A	FISH +
34	4n	FISH +
35	HP	FISH +
36	2n+LP (3n)	FISH -
38	LP (3n)	FISH -
39	2n+LP (3n)	FISH -
40	HP	FISH +

Table 4.7. *EGFR* gene status in SBA evaluable cases. FISH positive cases corresponding to a abnormal *EGFR* gene status are reported in red.
Legend: 2n = disomy; 3n = trisomy; 4n = tetrasomy; a = gene amplification; FISH- = FISH negative; FISH+ = positive; HP = high polysomy; LP = low polysomy; PT = patient identification.

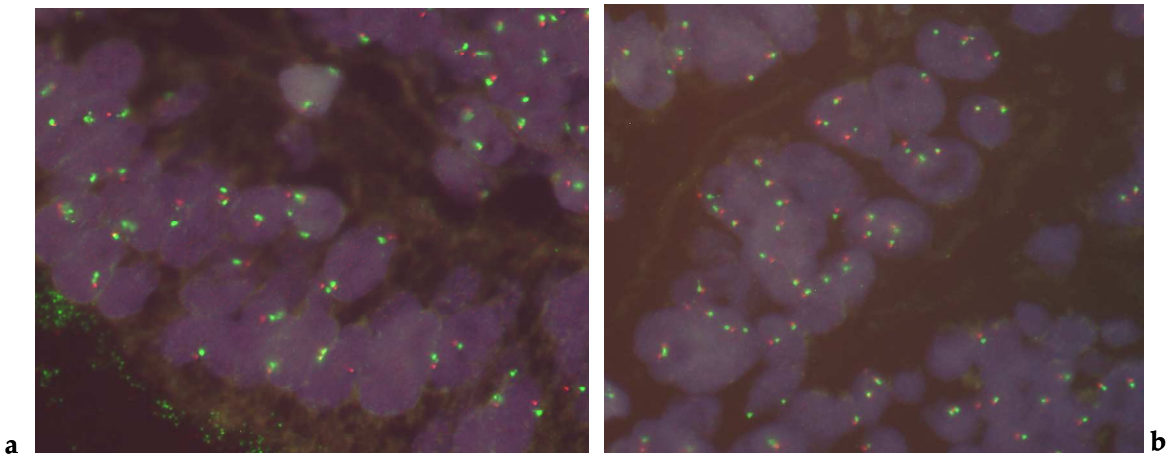

Figure 4.3. Example of *EGFR* gene status evaluation by FISH in SBA. **a:** disomy (2n); **b:** low polysomy (LP, trisomy). Red signals correspond to the *EGFR* gene; green signals correspond to the centromere of Chromosome 7.

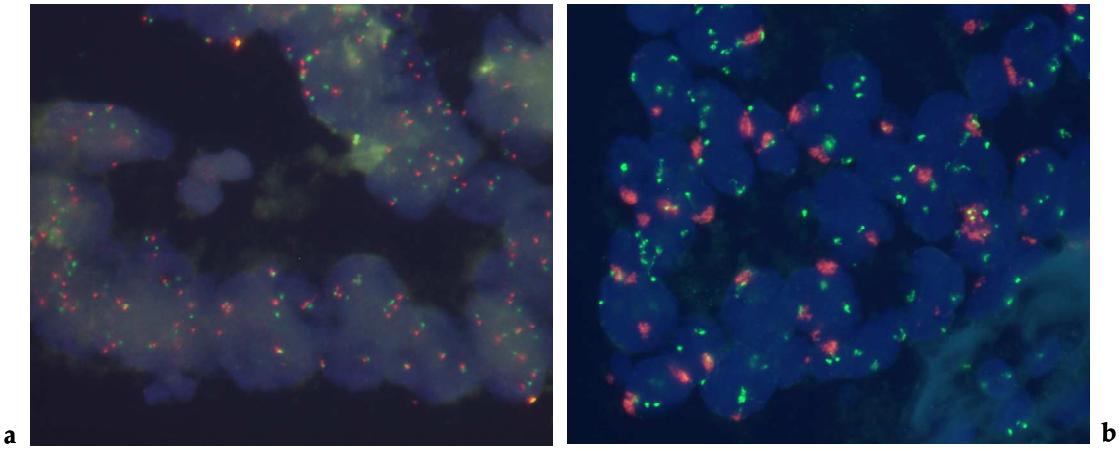

Figure 4.4. Example of *EGFR* gene status evaluation by FISH in SBA. **a:** high polysomy (HP); **b:** gene amplification. Red signals correspond to the *EGFR* gene; green signals correspond to the centromere of Chromosome 7.

4.8 *BRAF* gene mutations

BRAF mutations were investigated in exon 15 in 39 patients. Thirty-eight cases showed a wild-type sequence. One patient (PT = 18, Table 4.8) displayed a missense point mutation, leading to the G596R amino acid change. This patient was also characterized by MSS in the loci of the Bethesda panel (Table 4.3) and by absence of *KRAS* mutations (Table 4.4). No *BRAF* V600E mutations were observed. No *BRAF* mutations were observed in MSI cases.

Overall, *BRAF* mutations were identified in 2.6% of our SBA.

PT id	*BRAF*
1	WT
2	WT
3	WT
4	WT
5	WT
6	WT
7	WT
8	WT
9	WT
10	WT
11	WT
13	WT
14	WT
15	WT
16	WT
17	WT
18	G596R
19	WT
20	WT
21	WT

PT id	*BRAF*
22	WT
23	WT
24	WT
25	WT
26	WT
27	WT
28	WT
29	WT
30	WT
31	WT
32	WT
33	WT
34	WT
35	WT
36	WT
37	WT
38	WT
39	WT
40	WT

Table 4.8. Mutational status of SBA evaluable cases of *BRAF* gene (exon 15), by direct sequencing. Cases carrying a mutation are reported in red
Legend: PT id = patient identification; WT = wild-type. In the mutant cases, numbers identify the altered codon, the letter in the left of the number represents the wild-type amino acid sequence, the letter in the right of the number represents the altered amino acid.

4.9 *PIK3CA* gene mutations

PIK3CA mutations were investigated in exons 9 and 20 in 39 patients. Thirty-five cases showed a wild-type sequence (Table 4.9).

Four patients showed PIK3CA mutations, in particular:

- Exon 9: 2 missense point mutations, leading to E542K aminoacid change in 1 case (PT = 10, Table 4.9), and to Q546P change in 1 case (PT = 21, Table 4.9);
- Exon 20: 2 missense point mutations, leading to H1047R aminoacid change in 1 case (PT = 19, Table 4.9), and to G1049S change in 1 case (PT = 11, Table 4.9);

No mutations were found at codon 545 of exon 9, which represents the most altered codon in that exon.

Overall, *PIK3CA* mutations were identified in 10.2% of our cohort of SBA.

PT id	*PIK3CA*
1	WT
2	WT
3	WT
4	WT
5	WT
6	WT
7	WT
8	WT
9	WT
10	E542K
11	G1049S
13	WT
14	WT
15	WT
16	WT
17	WT
18	WT
19	H1047R
20	WT
21	Q546P

PT id	*PIK3CA*
22	WT
23	WT
24	WT
25	WT
26	WT
27	WT
28	WT
29	WT
30	WT
31	WT
32	WT
33	WT
34	WT
35	WT
36	WT
37	WT
38	WT
39	WT
40	WT

Table 4.9. Mutational status of SBA evaluable cases of *PIK3CA* gene (exons 9 and 20), by direct sequencing. Cases carrying a mutation are reported in red.
Legend: PT id = patient identification; WT = wild-type. In the mutant cases, numbers identify the altered codon, the letter in the left of the number represents the wild-type amino acid sequence, the letter in the right of the number represents the altered amino acid.

4.10 PTEN protein expression

The immunohistochemical evaluation of PTEN protein was performed in 39 cases (Table 4.10). Twenty-nine patients showed a PTEN expression similar to that observed in normal healthy mucosa (PTEN positive) (Figure 4.5), while 10 patients (PT = 3, 9, 14, 15, 16, 17, 18, 29, 34 and 38, Table 4.10) showed a loss of PTEN protein expression (i.e.: negative expression).

Overall, 25.6% of SBA showed loss of PTEN protein expression.

PT id	PTEN
1	Pos
2	Pos
3	Neg
4	Pos
5	Pos
6	Pos
7	Pos
8	Pos
9	Neg
10	Pos
11	Pos
13	Pos
14	Neg
15	Neg
16	Neg
17	Neg
18	Neg
19	Pos
20	Pos
21	Pos

PT id	PTEN
22	Pos
23	Pos
24	Pos
25	Pos
26	Pos
27	Pos
28	Pos
29	Neg
30	Pos
31	Pos
32	Pos
33	Pos
34	Neg
35	Pos
36	Pos
37	Pos
38	Neg
39	Pos
40	Pos

Table 4.10. PTEN protein expression in SBA evaluable cases, by immunohistochemistry. Negative cases corresponding to an abnormal protein expression are reported in red.
Legend: Neg = negative (abnormal expression); Pos = positive (normal expression) PT id = patient identification; WT = wild-type.

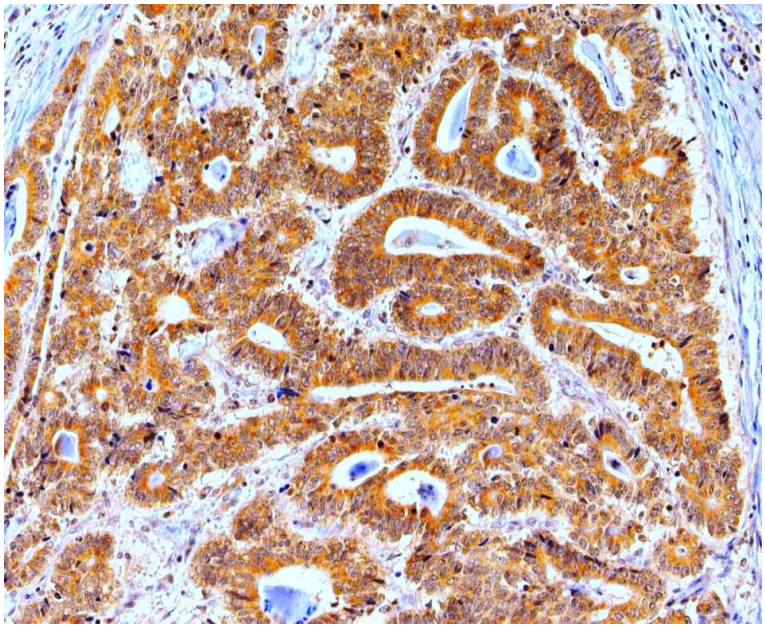

Figure 4.19. Example of normal expression of PTEN protein in SBA.

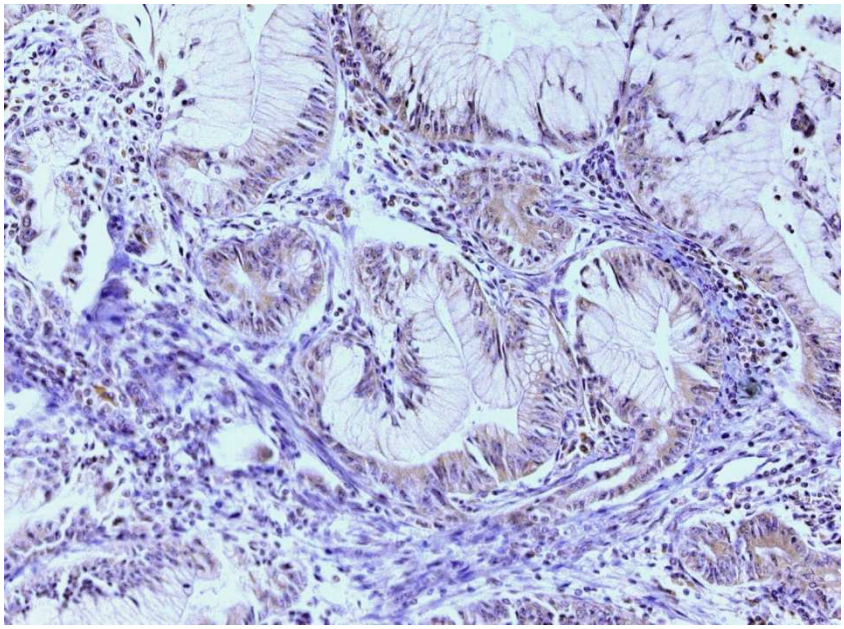

Figure 4.20. Example of loss of PTEN protein expression in SBA.

4.11 Correlations

By matching clinico-pathological and immunohistochemical-cytogenetic-molecular data, we observed several interesting correlations in our cohort of SBA.

- When we matched the single clinico-pathological parameters (sex, age at diagnosis, tumor location, tumor stage, tumor grade) with all the molecular alterations investigated (*KRAS*, *TP53*, *BRAF* and *PIK3CA* gene mutations, allelic imbalance of Chromosome 18q, MSI, β–catenin and PTEN protein expression, *EGFR* gene status) we found a significant correlation between *TP53* mutational status and tumor location. In fact, *TP53* mutations occurred more frequently in tumors arising in the jejunum than in those of the duodenum or ileum (p=0.02) (Tables 4.11 and 4.12). All the other correlations were not statistically significant.

		SEX		p	AGE		p	LOCATION			p
		M	F		≤ 60	> 60		Jejunum	Ileum	Duodenum	
KRAS	WT	13	9	**0,2**	4	18	**1**	7	6	8	**0,24**
	Mut	6	11		3	14		3	10	5	
TP53	WT	16	12	**0,155**	4	24	**0,37**	4	12	12	**0,02**
	Mut	3	8		3	8		6	4	1	
Microsatellite	MSI	13	16	**0,708**	5	24	**0,9**	6	14	9	**0,31**
	MSS	5	4		1	8		4	2	3	
Chr 18q	L	7	12	**0,35**	5	14	**0,2**	6	10	3	**0,15**
	NL	4	2		0	6		0	3	3	
BRAF	WT	19	19	**1**	6	32	**0,17**	9	16	13	**0,26**
	Mut	0	1		1	0		1	0	0	
PIK3CA	WT	17	15	**0,66**	6	28	**1**	8	13	13	**0,28**
	Mut	2	4		0	4		2	3	0	
PTEN	Pos	15	13	**1,714**	5	24	**0,9**	7	14	8	**0,26**
	Neg	4	6		2	8		3	2	5	
β-Catenin	Pos C	10	8		3	15		5	7	**6**	
	Pos N	4	6	**0,7**	1	8	**0,99**	1	7	1	**0,14**
	Neg	6	5		2	9		4	2	5	
EGFR	Pos	7	12	**1**	11	8	**0,732**	7	7	5	**0,609**
	Neg	5	7		7	7		3	7	4	

Table 4.11. Correlations among clinico-pathological features and immunohistochemical-cytogenetic-molecular data in SBA. p values are calculated using the two-tailed Fisher's exact test. Significant correlations ($p < 0.05$) are reported in red.
Legend: C = cytoplasm; Chr = chromosome; L = loss-of-heterozygosity; Mut = mutated; N = nucleus; Neg = negative; NL = non loss (absence of loss-of-heterozygosity); Pos = positive; WT = wild-type.

		STAGE								GRADE			
		T1	T2	T3	T4	p	N0	N1-N2	M1	P	G1	G2	G3	p
KRAS	WT	1	0	15	4	**0,2**	10	7	1	**0,5**	2	12	8	**0,28**
	Mut	0	2	8	5		5	7	2		1	13	2	
TP53	WT	1	2	14	6	**0,9**	9	10	2	**0,5**	2	17	5	**0,28**
	Mut	0	0	8	3		6	3	2		1	5	5	
Microsatellite	MSI	1	2	15	9	**0,2**	12	11	3	**0,9**	3	20	7	**0,69**
	MSS	0	0	8	0		3	4	1		0	5	3	
Chr 18q	L	1	2	10	5	**0,2**	8	6	2	**0,8**	3	12	4	**0,2**
	NL	0	0	13	4		7	9	2		0	13	6	
BRAF	WT	1	2	22	9	**1**	14	15	4	**0,14**	3	24	10	**1**
	Mut	0	0	1	0		0	0	1		0	1	0	
PIK3CA	WT	1	2	20	7	**0,8**	14	11	3	**0,34**	2	23	8	**0,29**
	Mut	0	0	3	2		1	4	1		1	2	2	
PTEN	Pos	1	2	20	6	**0,5**	12	12	3	**0,99**	3	17	8	**0,6**
	Neg	0	0	3	3		3	3	1		0	8	2	
β-Catenin	Pos C	1	1	11	3		8	7	2		1	14	3	
	Pos N	0	1	5	3	**0,79**	5	2	2	**0.29**	2	5	2	**0,17**
	Neg	0	0	7	3		2	7	1		0	5	5	
EGFR	Pos	0	1	12	5	**1**	9	6	2	**0,58**	2	11	5	**0,6**
	Neg	0	1	7	3		4	6	2		0	12	4	

Table 4.12. Correlations among clinico-pathological features and immunohistochemical-cytogenetic-molecular data in SBA. p values are calculated using the two-tailed Fisher's exact test. Significance is set up at $p < 0.05$.
Legend: C = cytoplasm; Chr = chromosome; L = loss of heterozygosity; Mut = mutated; N = nucleus; Neg = negative; NL = non loss (absence of loss of heterozygosity); Pos = positive; WT = wild-type.

- Then, by correlating β–catenin protein expression with MSI (representing the two different models of colorectal carcinogenesis), we found that this association was statistically significant. In fact, patients with MSI were characterized by absence of β–catenin overexpression (p=0.001) (Table 4.13).

		β-Catenin			p
		Pos C	Pos N	Neg	
Microsatellite	MSS	16	9	4	**0,01**
	MSI	2	0	7	

Table 4.13. Correlation between β-catenin protein expression and microsatellite instability in SBA. p values are calculated using the two-tailed Fisher's exact test. Significant correlation ($p < 0.05$) are reported in red.
Legend: C = cytoplasm; MSI = microsatellite instability; MSS = microsatellite stability; N = nucleus; Neg = negative; Pos = positive.

- Then, we compared the *EGFR* gene status with all the other molecular alterations and we found a statistically significant association between FISH positivity and the status of microsatellite loci included in the panel of Bethesda. In fact, a FISH positive pattern was more frequently found in MSS patients (p=0.004) (Table 4.14). In addition, we observed trends (but not statistically significant) between FISH positivity and the loss of heterozigosity (L) of Chromosome 18q (the majority of patients with Chromosome 18q loss are FISH positive for *EGFR*) (p=0.08) or with *KRAS* mutations (the majority of *KRAS* mutant cases are FISH positive for *EGFR*) (p=0.079) (Table 4.14).

		EGFR		p
		Pos	Neg	
KRAS	WT	12	4	**0,079**
	Mut	7	10	
TP53	WT	11	11	**0,28**
	Mut	8	3	
Microsatellite	MSS	18	7	**0,004**
	MSI	1	7	
Chr 18q	L	15	3	**0.08**
	NL	2	3	
BRAF	WT	18	14	**1**
	Mut	1	0	
PIK3CA	WT	16	12	**1**
	Mut	3	2	
PTEN	Pos	15	9	**0,44**
	Neg	4	5	
β-Catenin	Pos C	9	5	
	Pos N	6	2	**0,3**
	Neg	4	6	

Table 4.14. Correlation among *EGFR* gene status by FISH and immunohistochemical-molecular data in SBA. p values are calculated using the two-tailed Fisher's exact test. Significant correlations ($p < 0.05$) are reported in red.
Legend: C = cytoplasm; Chr = chromosome; L = loss of heterozygosity; Mut = mutated; N = nucleus; Neg = negative; NL = non loss (absence of loss of heterozygosity); Pos = positive; WT = wild-type.

- Finally, we stratified the patients on the basis of the molecular alterations involved in colorectal carcinogenetic models (*KRAS* and *TP53* mutations, allelic imbalance of Chromosome 18q) in MSS cases (because MSI cases follow a different mechanism of cancerogenesis). In this subgroup of 25 patients, 36% of cases was characterized by alteration of a single marker (*KRAS* mutations in 3 cases, 12%; *TP53* mutations in 1 case, 4%; Chromosome 18q loss in 5 cases, 20%). Twenty percent of patients showed alterations of both *TP53* and Chromosome 18q (5 cases), whereas 24% of patients both at *KRAS* and

Chromosome 18q level (6 cases). All the 3 molecular alterations were observed in 3 cases (12%). In our cohort of SBA, the concomitant alteration of *KRAS* and *TP53* was not identified. Finally, 8% of patients carried none of the three molecular alterations taken into account for this analysis (Figure 4.21).

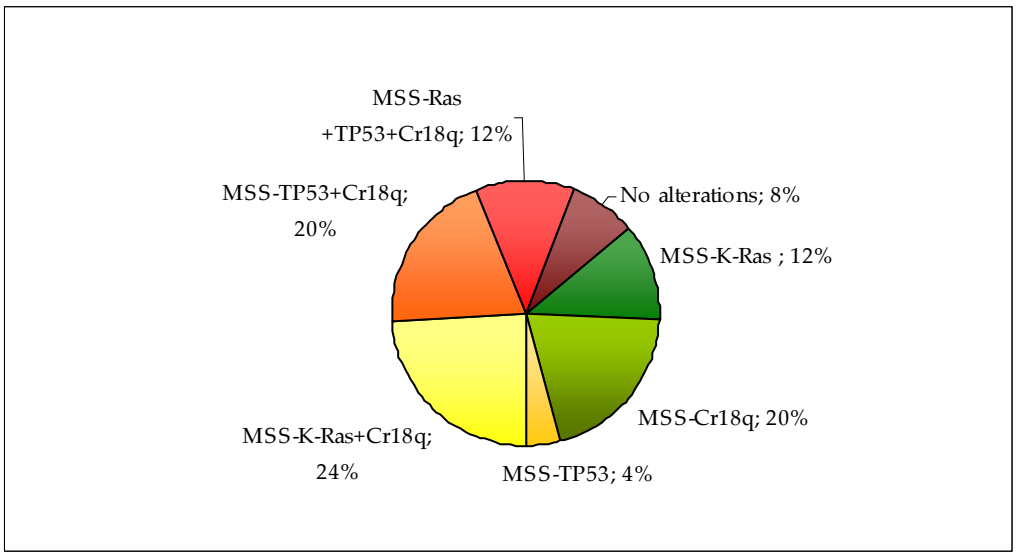

Figure 4.21. Subgroup of patients on the basis of *KRAS* mutations, allelic imbalance of Chromosome 18q and *TP53* mutations in SBA cases characterized by MSS.
Legend: Chr18q: Chromosome 18q.

By comparing these subgroups with tumor stage, we observed that the 2 metastatic cases with MSS feature were characterized by the simultaneous occurrence of *KRAS* and *TP53* mutations, and by Chromosome 18q loss. In addition, all the 3 cases showing the concomitant alteration of the 3 markers were classified at pT4 stage.

- When we compared the alterations occurring in EGFR downstream pathways (*KRAS*, *BRAF* and *PIK3CA* mutations, and PTEN protein expression) in patients characterized by a copy number gain of *EGFR* gene (i.e.: FISH positive cases) (19 patients), in 10 cases we observed at least 1 alteration in EGFR downstream members (4 with alteration at *KRAS* level, 2 with PTEN negative expression, 2 with mutations in both *KRAS* and *PIK3CA* genes, 1 with

KRAS mutation and PTEN loss of expression, 1 with *BRAF* mutation and PTEN loss of expression) (Table 4.15). Therefore, 9 patients were characterized by an alteration of the EGFR pathway limited to a copy number gain of *EGFR*, and absence of any other alteration in EGFR downstream pathways.

PT id	*EGFR*	*K-Ras*	*BRAF*	*PIK3CA*	PTEN
2	FISH +	WT	WT	WT	pos
3	FISH +	WT	WT	WT	pos
8	FISH +	WT	WT	WT	pos
11	FISH +	G12A	WT	G1049S	pos
14	FISH +	G12R	WT	WT	neg
16	FISH +	WT	WT	WT	neg
18	FISH +	WT	G596R	WT	neg
19	FISH +	G12D	WT	H1047R	pos
20	FISH +	G12V	WT	WT	pos
22	FISH +	WT	WT	WT	pos
24	FISH +	WT	WT	WT	pos
26	FISH +	WT	WT	WT	pos
27	FISH +	G12V	WT	WT	pos
31	FISH +	WT	WT	WT	pos
32	FISH +	WT	WT	WT	pos
33	FISH +	WT	WT	WT	pos
34	FISH +	WT	WT	WT	neg
35	FISH +	G12D	WT	WT	pos
40	FISH +	G12D	WT	WT	pos

Table 4.15. Cumulative analysis of EGFR pathways in *EGFR* FISH positive cases of SBA. Cases carrying an alteration in EGFR downstream pathways are reported in red.
Legend: neg = negative; pos = positive; PT id = patient identification; WT = wild-type. In the mutant cases, numbers identify the altered codon, the letter in the left of the number represents the wild-type amino acid sequence, the letter in the right of the number represents the altered amino acid.

4.12 Correlation of molecular data with clinical response to EGFR-targeted therapy

Patient #3 (Table 4.1) developed an aggressive disease and was addressed to the administration of EGFR-targeted therapies. More in details, when he was 48, the patient complained of abdominal pain and vomiting referable to intestinal obstruction. An abdominal CT scan showed multiple liver metastasis and peritoneal carcinomatosis. The explorative laparotomy revealed a tumor mass of the ileum

consistent with an adenocarcinoma, and a palliative resection was performed. So the distant (and very spread) metastatic lesions were synchronous with the discovery of the primary tumor. Chemotherapy with oxaliplatin and capecitabine was administered for 10 months, and a PR was achieved as best response. Due to PD, the FOLFIRI regimen was prescribed for 8 months, and a PR was obtained. Then the patient experienced weight loss, abdominal pain and subocclusive bowel episodes due to progression of abdominal metastatic sites. Based on good performance status (PS), disabling tumor-related symptoms and absence of standard therapeutic options, the molecular characterization of the EGFR pathway was performed. The patient turned out to show *EGFR* copy number gain (FISH positive) and absence of any alterations in EGFR downstream pathways (i.e.: *KRAS* wild-type, *BRAF* wild-type, *PIK3CA* wild-type, and PTEN positive expression). After informed consent, cetuximab was administered in combination with irinotecan. The treatment was relatively well tolerated, and only a grade 2 cutaneous rash developed. After 8 weeks, the CT scan documented a disease stabilization, with a rapid and dramatic relief of symptoms (pain improvement according to the Visual Analogue Scale and reduced analgesic consumption). Unfortunately, after 5 months of therapy, the patient experienced obstructive abdominal symptoms with deterioration of PS. Consequently he was referred to best supportive care, and death occurred due to PD.

DISCUSSION

SBA is a rare but aggressive disease. Due to its rarity, only few studies have investigated this neoplastic disease, and several aspects still need to be elucidated: for example, it is not known if patients characterized by a tumor with regional lymph nodes invaded by metastatic cells, has a worse prognosis than patients without metastatic disease. Risk factors and histological appearance of SBA are superimposable with those of colorectal cancers (Delaunoit et al, 2004; Pan et al, 2011). This finding has led clinicians to the treatment of SBA patients with protocols set-up for colorectal adenocarcinomas.

At molecular level, at odds with colorectal cancer which has been the object of extensive investigation, SBA is very poorly characterized. For colorectal cancers, two models of cancerogenesis have been proposed: the first one is characterized by MSI and is valid for a subgroup (10-15% of cases) of sporadic cases as well as for patients belonging to HNPCC families (Ilyas et al, 1999; Houlston et al, 2001); the second one is characterized by the sequential occurrence of alterations in *APC*, *KRAS*, tumor suppressor genes located on the long arm of Chromosome 18q, and *TP53*, and is valid for the vast majority of sporadic cases as well as for patients belonging to FAP families (Laurent-Puig et al, 1999; Fearon and Vogelstein, 1990).

Very few studies have investigated SBA at molecular level. Generally the markers involved in the two models proposed for colorectal carcinogenesis have been studied in SBA, but not in an organic way. Indeed, the studies published so far investigated only few markers. Despite these limitations, on the basis of the similar percentages of alterations of the aforementioned markers observed in SBA with respect to colorectal cancer, it has been proposed that the models of carcinogenesis proposed for colorectal cancer are valid for SBA too.

To demonstrate in a clearer manner if the assumption that SBA carcinogenesis is superimposable with that of colorectal cancer (thus better justifying that SBA patients can be treated with chemotherapies set-up and validated for colorectal cancer patients), we decided to investigate all the aforementioned markers in the same cohort of SBA. To do this we recruited tissues from different institutions: the Institute

of Pathology in Locarno and 3 institutions from Northern Italy. We excluded cases for which the duodenal origin was doubtful (i.e.: cases in which the tumor was diffused also in the pancreas and the histological analysis showed a poorly differentiated aspect) and tumors of the papilla of Vater, in order to have an homogeneous cohort (cases of Papilla of Vater have pancreatic features and the percentage of *KRAS* mutations is increased with respect to SBA). Our cohort had clinical characteristics similar to those observed in the studies already published, with a little prevalence of tumors arising in the ileum (40%) with respect to those occurred in the duodenum (35%). More than 80% of cases were at advanced stage (60% pT3 and 22.5% pT4), and about half of patients displayed the presence of metastatic cells at least in regional lymph nodes (41.6%).

Percentages and type of alterations in our cohort were superimposable with those reported in the literature concerning SBA studies (http://www.sanger.ac.uk/genetics/CGP/cosmic; Bläker et al, 2002; Planck et al 2003; Delaunoit et al, 2005; Overman et al, 2010).

When we matched the results obtained in our cohort of SBA with those published in colorectal cancer (and also with unpublished results on colorectal cancer obtained in our laboratory), we observed a quite high rate of MSI (23.6% of cases). Usually, in colorectal cancer the percentage of MSI cases is around 10-15%, but there are also some cohorts with a rate of MSI higher than that range (Bläker et al, 2002; Markowitz and Bertagnolli, 2009), so we can conclude that a superimposable number of SBA and colorectal cancer follow the MSI-based model of cancerogenesis.

As for the analysis of the APC-β-catenin pathway alterations, we focused our attention on the evaluation of β-catenin protein expression. Deregulation of the APC-β-catenin pathway (due to either *APC* mutations, or *APC* loss-of-heterozigosity, or *APC* promoter hypermethylation, or *β-catenin* mutations) leads, in fact, to β-catenin accumulation, mainly in the nucleus but also in the cytoplasm, detectable by an increase of β-catenin expression in IHC. In our cohort of SBA, only 23.6% of cases showed β-catenin expression at nuclear level, and additional 47.3% showed β-catenin

overexpression only at cytoplasmic level, thus confirming a previous report (Overman et al, 2010). However, we should note that if *APC* mutations are detected in the vast majority of cases of colorectal cancer (more than 80% of sporadic cases), the reported rates for β-catenin expression in colorectal cancer range from 20 to 100%, thus showing that there is not 100% concordance between altered levels of β-catenin expression and *APC* alterations. This fact could be due to the difficulties in the evaluation of this marker. The results of our cohort, therefore, are consistent with those of colorectal cancer (Frattini et al, 2004). Additionally, by comparing microsatellite status and β-catenin expression, we found that the two alterations, which are alternatively altered in colorectal cancer, are significantly associated with different mechanisms of cancerogenesis also in SBA.

As for *KRAS*, we found a point mutations in about 40% of cases, mainly at codon 12. The percentage and the types of alterations in codons 12 and 61 mirror those reported for colorectal cancer (Markowitz and Bertagnolli, 2009). However, in colorectal cancer, a not negligible fraction of cases (about 20% of those carrying a *KRAS* mutation) show a mutation at codon 13 (the G13D change in nearly the totality of cases). This datum is discrepant with our cohort of SBA, where no cases with *KRAS* codon 13 mutations were observed. A possible explanation of this finding is as follow. Although the prognostic role of *KRAS* mutations has never been confirmed, it has been proposed that specific mutations might be associated with different tumor behaviour. Finkelstein and colleagues, two decades ago, proposed in particular that codon 13 mutations might be associated with an indolent phenotype of the tumor, whereas mutations occurring at codon 12 might be associated with a more aggressive disease (Finkelstein et al, 1993). This hypothesis was sustained by a recent work of Zlobec and colleagues, who demonstrated that patients with *KRAS* codon 13 mutations experience a better prognosis than those with a *KRAS* codon 12 alteration (Zlobec et al, 2010). Ninety percent of our cohort of SBA was represented by T3/T4 stage tumors, a datum which can indirectly justify the absence of KRAS codon 13 mutations in our cohort of SBA.

As regards the allelic imbalance of Chromosome 18q, the percentage of deregulation found in the cohort of SBA is in the range observed in colorectal cancer (Markowitz and Bertagnolli, 2009). The same is true also for *TP53* mutations (for both percentage of alterations and types of mutations), although the percentage of mutations in our cohort of SBA is quite low, but still in the range of colorectal cancer (Markowitz and Bertagnolli, 2009).

Focusing the attention on MSS cases where the analyses of allelic imbalance of Chromosome 18q could be performed, and excluding β-catenin expression (in agreement with a similar work appeared on colorectal cancer) (Frattini et al, 2004), we observed the following patterns of molecular alterations:

- 8% of cases: no alterations found;
- 36% of cases: only 1 alteration (*KRAS*: 12%, Chromosome 18q: 20%; *TP53*: 4%);
- 44% of cases: 2 alterations (Chromosome 18q+*TP53*: 20%; *KRAS*+*TP53*: 24%);
- 12% of cases: 3 alterations (*KRAS*+Chromosome 18q+TP53).

These combinations of alterations are superimposable with those obtained by the analysis of a cohort of colorectal cancer (Frattini et al, 2004). It is noteworthy that all the 3 cases with 3 alterations (*KRAS*+Chromosome 18q+*TP53*) were classified as T4 and, in 2 cases, a distant metastatic lesion was also present at the time of first diagnosis, thus confirming the association between the accumulation of genetic alterations in the markers involved in the Vogelstein's models, with tumor progression and, in particular, with late stages of carcinogenesis.

In conclusion, the analyses of the markers playing a pivotal role in the two carcinogenetic models proposed by Vogelstein's group in colorectal cancer, indicate a superimposable pattern of alteration (both for percentages and types of mutations) between SBA and colorectal cancer. Our data reinforce therefore the choice of oncologists to treat SBA patients with protocols valid for adenocarcinoma of the large intestine.

In colorectal cancer, the analysis of all the aforementioned markers have not been introduced in clinical practice (excluding MSI, used in diagnosis for the identification of patients belonging to HNPCC families), whereas a new pathway, the EGFR pathway, has acquired high clinical relevance. Therefore, the demonstration that SBA and colorectal cancer share the same molecular alterations and the same cancerogenetic development, led us to investigate the EGFR pathway in SBA, with the goal of the introduction of new therapies in SBA management. In colorectal cancer, in fact, EGFR-targeted therapies represent a success story, because metastatic patients treated with these compounds experienced longer overall survival with respect to patients treated with classical chemotherapies only. However, it has been demonstrated that only a subgroup of patients may be sensitive to these therapies, and that a careful molecular evaluation of patient's tumors may predict the efficacy of EGFR-targeted therapies.

In the literature, Overman and colleagues demonstrated EGFR overexpression at protein level in 70% of SBA (Overman et al, 2010), a percentage in keeping with colorectal cancer data. However, in adenocarcinoma of the large intestine it has been shown that immunohistochemistry is not a gold standard method to evaluate EGFR, because the type of fixative used, the storage time of unstained tissue sections (Atkins et al, 2004), the type of primary antibody used (Kersting et al, 2006) and the methods of IHC evaluation (Langner et al, 2004) might generate conflicting data in the EGFR assessment.

However, it has been proposed that *EGFR* gene copy number gain (analyzed by FISH) may represent an efficient marker to identify patients who can benefit from EGFR-targeted therapies administration (Moroni et al, 2005; Lièvre et al, 2006, Frattini et al, 2007; Sartore-Bianchi et al, 2007; Cappuzzo et al, 2008). In our cohort of SBA, an *EGFR* gene copy number gain was observed in 57.5% of cases, a percentage in keeping with the literature of colorectal cancer (Frattini et al, 2007; Martin et al, 2009). This group may therefore include the patients to be addressed to the administration of EGFR-targeted therapies. However, a recent ring test among

several laboratories with high experience in *EGFR* FISH evaluation, has demonstrated that such a methodology suffers from inter-observer variability (Sartore-Bianchi et al, 2012), preventing, therefore, the use of such a marker for the selection of patients to be addressed to EGFR MoAb administration.

A well accepted marker useful for early identification of the efficacy of EGFR-targeted therapies is represented by the occurrence of *KRAS* mutations (Custodio et al, 2013). In particular, FDA and EMA guidelines indicate that only patients with a *KRAS* wild-type sequence can be addressed to EGFR MoAb treatment. With this simple and fast test, about 30-40% of cases who are naturally resistant to anti-EGFR compounds (due to the presence of *KRAS* mutation) can be excluded from the administration of such therapies and addressed to more appropriate chemotherapeutic combinations. As for *BRAF*, *PIK3CA* and PTEN deregulations, however, the information on the effect of these alterations on the efficacy of EGFR-targeted therapies in advanced colorectal cancer patients are not completely confirmed. The majority of studies (reviewed by Custodio et al, 2013) indicate that the *BRAF* V600E change, as well as *PIK3CA* exon 20 mutations and the loss of expression of PTEN may represent alternative negative predictors of the efficacy of EGFR-targeted therapies. However these tests have not entered in clinical practice, even if it was reported that the combination of all these analyses may increase the possibility to early identify patients resistant to EGFR MoAbs (Frattini et al, 2007; Sartore-Bianchi et al, 2010). Overall, in our cohort of SBA, if we look only at *KRAS* mutations, we can propose the administration of EGFR-targeted therapies to about 60% of patients (i.e.: only on *KRAS* wild-type cases), and to 23% of cases if we base our evaluation on the whole EGFR pathway (i.e.: only on cases showing, at the same, time, *EGFR* copy number gain, *BRAF* and *PIK3CA* wild-type sequence, and PTEN normal expression). However, as for colorectal cancer, these hypotheses must be confirmed at clinical level, with ad hoc clinical trials where the molecular characterization is performed on treated patients.

Although these data are missing for SBA, at the moment, oncologists at the Oncology Institute of Southern Switzerland (Bellinzona), starting from the similarity at molecular level of SBA and CRC, decided to treat a SBA patient with advanced disease with EGFR-targeted therapies (i.e.: cetuximab). Our case was the first report of the use of an anti-EGFR MoAb in combination with chemotherapy in advanced SBA. After adding cetuximab to irinotecan as third line regimen, the patient experienced disease stabilization with translated clinical benefit. At molecular level, the patient showed *EGFR* gene copy number gain and absence of any alterations in EGFR downstream pathways (in particular, the *KRAS* gene was wild type for codons 12, 13 and 61). This finding, although anecdotic, confirm in SBA the results obtained by our laboratory in colorectal cancer patients treated with EGFR-targeted therapies (Frattini et al, 2007; Di Nicolantonio et al, 2008; Sartore-Bianchi et al, 2009) and reinforce the notion that a better understanding of molecular alterations characterizing SBA is mandatory to early identify patients to be addressed to EGFR MoAb. Our work was then supported by another study, where it has been reported reported 4 SBA patients treated with cetuximab, all showing disease stabilization, or partial or complete response (Santini et al, 2010). In three of these cases the *KRAS* gene status was investigated, and in all the cases the Authors did not find any *KRAS* mutation, thus confirming the negative predictive role of *KRAS* mutations in EGFR-targeted therapies, not only in colorectal cancer but also in SBA.

Finally, it should be mentioned that new markers playing a predictive role of the efficacy if EGFR MoAb have been recently introduced into clinical practice. These additional alterations are *KRAS* mutations not occurring at codons 12 and 13 (i.e.: at codons 59, 61, 117 and 146) and *NRAS* mutations. These markers have been approved by FDA only few months ago, and were not tested in our cohort of SBA. However, our work on SBA tumors will be followed by the extensive characterization with next generation sequencing technology (i.e.: Ion Torrent), using the Hot Spot Cancer Panel, which includes more than 700 mutations in 50 genes, and all *KRAS* and all *NRAS* mutations are included. The analyses are now on going.

By the analysis of EGFR pathway, we can observe four corollaries:

- the first one is represented by the correlations between *EGFR* gene status and the other molecular alterations. In our cohort of SBA, we found that *EGFR* gene copy number was associated with a MSS pattern and with loss-of-heterozigosity of Chromosome 18q. Since in colorectal cancer MSS and allelic imbalance of Chrsomome 18q are associated with worse prognosis, we can suggest that EGFR gene copy number gain may be associated with a more aggressive disease also in SBA.
- The *BRAF* mutation we observed (G596R) is very rare, reported only in few cases of colorectal cancer. In addition, it is interesting to note that we did not identify any V600E change, the typical *BRAF* mutation in colorectal cancer, especially in MSI cases. Our work is the first investigating *BRAF* in a SBA cohort, and therefore our data deserve confirmation. We can state that the type of *BRAF* mutation in SBA is different than those routinely observed in colorectal cancer.
- The third one is the confirmation also in SBA cases of the mutual exclusivity of *KRAS* and *BRAF* mutations, already reported for papillary thyroid carcinoma (Frattini et al, 2004) in addition to colorectal cancer (Rajagopalan et al, 2002; Lièvre et al, 2006; Di Nicolantonio et al, 2008), thus supporting the notion that these two genes play a superimposable role in cancer development.
- The last one is represented by the observation of the mutual exclusivity of *PIK3CA* mutations and PTEN loss of expression, already reported in colorectal (Frattini et al, 2005) and in breast carcinoma (Maurer et al, 2009). As for *BRAF* and *KRAS*, we can therefore hypothesize a superimposable role of *PIK3CA* and PTEN in cancer development

In conclusion, our analysis of SBA confirm the feeling that the mechanisms of carcinogenesis of such disease are superimposable with those proposed for colorectal

cancer. Therefore the hypothesis that therapeutic protocols valid for the large intestine can be applied also to SBA patients is supported. As a consequence, the targeted therapies recently introduced in colorectal cancer can be proposed also for SBA patients. On this regard, by reporting a case report, we strongly suggest a possible role of cetuximab in the management of SBA. Nonetheless, prospective controlled trials to ascertain the role of anti-EGFR MoAbs in SBA are warranted. Finally, the application in the near future of next generation sequencing technology will significantly increase the molecular knowledge of SBA and, possibly, will identify new alterations in markers potentially druggable by new targeted therapies.

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